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Décoloration d'effluents de distillerie par un consortium microbien

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DECOLORIZATION OF MOLASSES WASTEWATER FROM DISTILLERIES USING BACTERIAL CONSORTIUM

Miss Suhuttaya Jiranuntipon

MOTS-CLES : décolorazation / molasses / effluents / consortium microbien / membrane bioreacteur / melanoidins

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Les effluents de distillerie de mélasse de canne à sucre génèrent une pollution environnementale due à, d'une part de grands volumes et d'autres part à la présence de composés de couleur brune foncée, connus sous le nom de mélanoïdines. Dans cette étude, un consortium bactérien CONS8 isolé dans des sédiments de chute d'eau a été choisi comme consortium apte à la décoloration de la mélasse. On a montré que le consortium CONS8 pouvait décolorer, trois eaux usées synthétiques différentes, élaborées respectivement à base de Viandox (13,48% v/v), d'eau usée de mélasse de betterave (41,5% v/v) ou d'eau usée de mélasse de canne à sucre (20% v/v). Les décolorations obtenues en 2 jours seulement, en fioles d'Erlenmeyer sont respectivement de 9,5, à 8,02 et à 17,5%. Quatre bactéries prédominantes ont été identifiées dans le consortium CONS8 par l'analyse de l'rADN 16S. Sur la base de cette identification, et afin de réaliser la décoloration la plus élevée, un consortium bactérien artificiel MMP1 a été reconstruit avec Klebsiella oxytoca, Serratia mercescens (T2) et la bactérie inconnue DQ817737 (T4). Dans des conditions optimisées (aération, pH) le consortium bactérien MMP1 a permis de décolorer l'eau usée synthétique contenant de la mélanoidine à 18,3% en 2 jours. La comparaison de la décoloration par le consortium MMP1 avec un milieu abiotique a démontré que la décoloration était principalement due à l'activité biotique des cellules bactériennes, sans aucun phénomène d'adsorption. Un complément en minéraux et vitamines B n'a pas amélioré la décoloration de mélanoïdines avec le consortium bactérien MMP1. Enfin, les performances d'un bioréacteur à membrane pour traiter les eaux résiduaires synthétiques contenant de la mélanoïdine ont été évaluées à l'échelle du laboratoire. L'ensemencement du réacteur a été réalisé avec un inoculum sur la base du consortium MMP1. Le réacteur a fonctionné sous plusieurs conditions de temps de séjour hydrauliques (HRT) de 15, 20, et 40 heures. Les performances ont été analysées en termes de DCO (demande chimique en oxygène), décoloration et croissance de biomasse. Les résultats ont indiqué qu'une efficacité accrue d'élimination de la DCO et de la couleur ont été obtenues avec le HRT le plus long.

KEYWORDS : DECOLORIZATION / MOLASSES / WASTEWATER / BACTERIAL CONSORTIUM / MEMBRANE BIOREACTOR

SUHUTTAYA JIRANUNTIPON : DECOLORIZATION OF MOLASSES WASTEWATER FROM DISTILLERIES USING BACTERIAL CONSORTIUM. ADVISOR : CLAIRE ALBASI, Dr. de l'INPT, CO-AVISOR : PROF. SOMSAK DAMRONGLERD, Dr.Ing., 183 pp.

Distillery effluent from sugarcane molasses leads to an environmental pollution due to its large volume and the presence of dark brown colored compounds, known as melanoidins. In this study, a bacterial consortium CONS8 isolated from waterfall sediments in Maehongsorn province was selected as a molasses-decolorizing consortium. Consortium CONS8 was able to decolorize, only within 2 days, in Erlenmeyer flasks, three different synthetic wastewaters containing either Viandox sauce (13.5% v/v), beet molasses wastewater (41.5% v/v) or sugarcane molasses wastewater (20% v/v) at 9.5, 8.0 and 17.5%, respectively. Four predominant bacteria present in the consortium CONS8 were identified by the 16S rDNA analysis. To achieve the highest decolorization, the artificial bacterial consortium MMP1 comprising *Klebsiella oxytoca*, *Serratia mercescens* (T2) and unknown bacterium DQ817737 (T4), was constructed. Under optimized conditions (aeration, pH), the bacterial consortium MMP1 was able to decolorize the synthetic melanoidins-containing wastewater at 18.3% within 2 days. The comparison of decolorization by the consortium MMP1 with abiotic control proved that the color removal for synthetic melanoidins-containing wastewater medium was mainly due to biotic activity of bacterial cells, without any adsorption phenomena. Supplement of nutrients and vitamin B did not promote melanoidins decolorization by bacterial consortium MMP1. Finally, the performance of a membrane bioreactor (MBR) for synthetic melanoidins-containing wastewater treatment was investigated at laboratory scale, with a mineral membrane. The reactor seeding was made with the MMP1 bacterial consortium inoculum. The reactor was performed with several hydraulic retention times (HRT) of 15, 20, and 40 hours. The performances were analyzed in terms of COD, color removal and biomass in the reactor. The results indicated that the higher COD and color removal efficiency were achieved with the longer HRT.

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CHAPTER I

INTRODUCTION

Various countries, such as Thailand, Malaysia, Taiwan and Brazil, produce sugar from cane. Many by-products from sugar cane process, such as molasses, bagasse and fiber cake, are produced (Sirianuntapiboon et al., 2004). Molasses is the more important by-product and it is used as carbon source in fermentations, biofertilizer and feed for domestic animal (Dahiya et al., 2001). Molasses is commonly used as raw material in fermentation industries, especially for ethanol production because of its low-cost, availability and suitability for fermentation process. However, wastewater from molasses processing presents high organic load causing a large chemical oxygen demand (COD) and a large amount of colored substances which give dark brown color, to the effluents. After the conventional biological treatment, most of the organic load is removed but nevertheless the dark brown color still persists, it can even increase because of the repolymerization of colored compounds. The main colored compounds are known as melanoidins formed via "Maillard reaction" which is initiated by the condensation of an amine with a carbonyl group, often from a reducing sugar (Peña et al., 2003). The structural determination of the melanoidins thus remained a challenge over many decades, with little progress made. However, there are currently three main proposals for the structure of melanoidins: (i) polymer consisting of repeated units of furans and/or pyrroles, formed during the early stages of Maillard reaction, and linked by polycondensation reactions; (ii) low-molecular-weight colored substances, which are able to cross-link protein via amino groups of lysine or arginine to produce high-molecular-weight colored melanoidins; and (iii) melanoidin skeleton is mainly built up of sugar degradation products, from the early stages of Maillard reaction, polymerized through aldol-type condensation, and linked by amino compounds.

Melanoidins are major pollutant when discharged into a water resource system. They prevent the penetration of sunlight and affect the photosynthetic activity of aquatic plants. The high organic load of the effluent causes eutrophication. This will therefore create anaerobic conditions thereby killing most of the aerobic aquatic fauna (Bernardo et al., 1997; Raghukumar et al., 2004). When spread over soil,

molasses wastewater acidifies soil and affects agricultural crops (Raghukumar et al., 2004).

In Thailand, almost of the alcohol-producing industries use molasses as raw material and discharge molasses wastewater accounting for about 10 times the amount of alcohol produced. Several alcohol distilleries in Thailand have attempted to treat molasses-based distillery wastewaters by anaerobic methods such as methane fermentation and waste stabilization pond (WSP) system followed by aerobic treatment such as activated sludge system, aerated lagoon or oxidation pond (Hammer, 1991). However, by these treatment processes, almost all of melanoidins in distillery wastewater still remained and the chemical oxygen demand (COD) of the treated wastewater is higher than the standard permission value of the Department of Industrial Works, Ministry of Industry, Thailand (Department of Industrial Works, 1992)

As melanoidins are recalcitrant to biodegradation, the elimination of colored effluents in molasses-based distillery wastewater treatment system is mainly based on physical or chemical procedures such as adsorption, coagulation, precipitation, and oxidation. Although these methods are effective, they suffer from such short coming as requiring high reagent dosage, high cost, formation of hazardous by-products and intensive energy consumption. These methods also generate large amount of sludge. Therefore, as an alternative, biological treatments with microbe are drawing attention.

The present study explores the feasibility of using bacterial isolates for molasses decolorization, with the ultimate aim of application for molasses-based distillery wastewater treatment. In this work, a bacterial consortium capable of decolorizing molasses wastewater was isolated from various sources in Thailand. The identification of bacterial strains by 16S rDNA based molecular approach was performed. Different parameters for maximal molasses wastewater decolorization were tested. The performance of a carbon membrane bioreactor (MBR) process for treating the molasses-based distillery wastewater by bacterial consortium was also investigated.

CHAPTER II

BIBLIOGRAPHY

2.1 Current production of sugarcane molasses in Thailand

Total Thai sugarcane production averaged 50–60 million tons a year from planted area of 1 million hectares (Office of Agriculture Economics [OAE], 2006). From this harvest, approximately 2.5–3 million tons of molasses is produced, 60-70% of which is locally utilized in many industries including food, feed and distillery, and the rest are supplied to export market (OAE, 2004). The increase in production of molasses in Thailand crops from 1988 and 2007 is shown in Table 1.

Table 1 Molasses production in Thailand for the crops years 1988/89-2006/07 (Office of the Cane and Sugar board [OCSB], 2007).

Crops Year	Molasses Production (million tons)				
	Northern	Central	Eastern	North Eastern	Total
1988/ 1989	0.324	0.981	0.204	0.242	1.742
1989/ 1990	0.288	0.645	0.208	0.278	1.719
1990/ 1991	0.480	1.091	0.197	0.400	2.168
1991/ 1992	0.555	1.169	0.208	0.470	2.402
1992/ 1993	0.411	0.743	0.151	0.318	1.623
1993/ 1994	0.455	0.870	0.157	0.436	1.918
1994/ 1995	0.618	1.069	0.182	0.767	2.636
1995/ 1996	0.703	1.028	0.220	0.902	2.853
1996/ 1997	0.638	1.044	0.156	0.756	2.594
1997/ 1998	0.503	0.705	0.116	0.894	2.218
1998/ 1999	0.502	0.862	0.148	0.833	2.396
1999/ 2000	0.497	0.855	0.153	0.917	2.421
2000/ 2001	0.480	0.823	0.141	0.823	2.266
2001/ 2002	0.557	0.958	0.190	1.098	2.803
2002/ 2003	0.670	1.178	0.208	1.480	3.536
2003/ 2004	0.668	1.024	0.161	1.062	2.915
2004/ 2005	0.509	0.784	0.132	0.824	3.249
2005/ 2006	0.520	0.786	0.115	0.688	2.110
2006/ 2007	0.760	1.047	0.157	1.035	2.999

Alcohol distilleries are considered as one of the largest polluter in Thailand. The effluents from such industry cause color problems, slime growth, thermal impacts, scum formation, and loss of aesthetic beauty in the environment. They also increase the amount of toxic substances in the water, causing death to the zooplankton and fishes, as well as profoundly affecting the terrestrial ecosystem.

The increasing public awareness of the fate of this pollutant and stringent regulations established by the various governmental authorities are forcing the industry to treat effluents to the required compliance level before discharging them into the environment.

2.2 Alcohol production from sugarcane molasses

Alcohol can be produced from a wide range of feedstock. These include sugar-based (sugarcane and beet molasses, cane juice), starch-based (corn, wheat, cassava, rice, barley) and cellulosic (crop residues, sugarcane bagasse, wood, municipal solid wastes) materials.

The production of alcohol in distilleries based on sugarcane molasses constitutes a major industry in Asia and South America. The world's total production of alcohol from sugarcane molasses is more than 13 millions m^3/year.

The manufacture of alcohol in distilleries consists of four main steps as follow: feed preparation, fermentation, distillation and packaging (Figure 1).

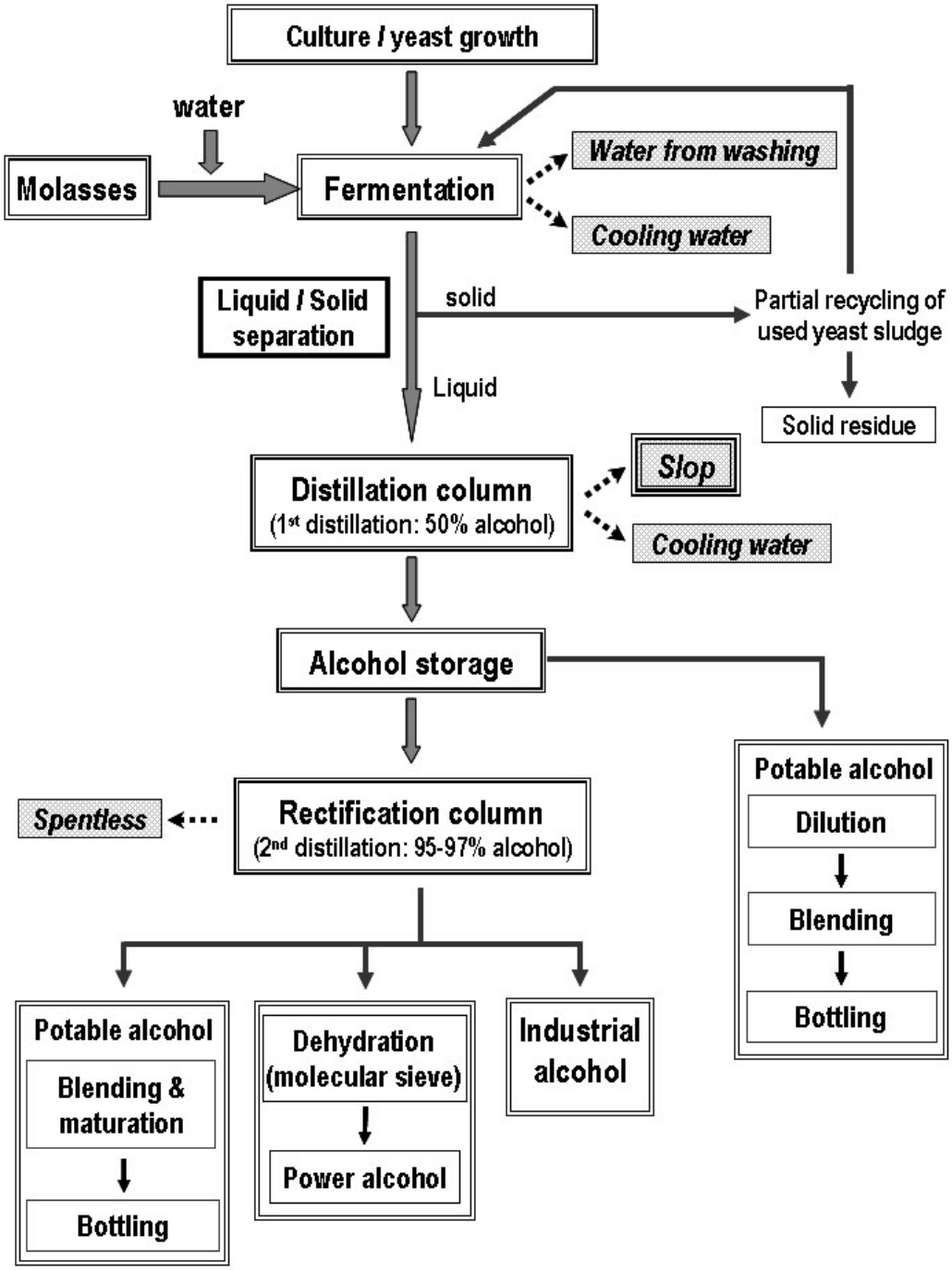

Figure 1 Alcohol manufacturing process (modified from Satyawali and Balakrishnan, 2008)

2.2.1 Feed preparation

Molasses is diluted to about 20–25 brix in order to obtain desired sucrose level and pH is adjusted to below 5 using sulfuric acid before fermentation. It is then supplemented with assimilable nitrogen source like ammonium sulfate or urea. If necessary, it is also supplemented with phosphate.

The composition of molasses varies with the variety of cane, the agro climatic conditions of the region, sugar manufacturing process, handling and storage (Godbole, 2002). Table 2 summarizes the chemical composition of sugarcane molasses.

Table 2 Composition of sugarcane molasses (Godbole, 2002; Chen and Chou, 1993).

Property	**Sugarcane molasses**	
	Godbole (2002)	Chen and Chou (1993)
Brix (%)	79.5	85 - 92
Specific gravity	1.41	1.38 - 1.52
Total solids (%)	75.0	75 - 88
Total sugar (%)	44 - 60	50 - 90
Crude protein (%)	3.0	2.5 – 4.5
Total fat (%)	0.0	0.0
Total fiber (%)	0.0	0.0
Ash (%)	8.1	7 - 15
Calcium (%)	0.8	NR
Phosphorus (%)	0.08	NR
Potassium (%)	2.4	NR
Sodium (%)	0.2	NR
Chlorine (%)	1.4	NR
Sulfur (%)	0.5	NR

NR: Not reported.

2.2.2 Fermentation

In general, yeast culture (*Saccharomyces cerevisiae*) is prepared in the laboratory and propagated in a series of fermenters. The feed is inoculated with about 10% by volume of yeast inoculum. This is an anaerobic process carried out under controlled conditions of temperature and pH. Sucrose is broken down to ethanol and carbon dioxide. Fermentation can be carried out in either batch or continuous mode. Ethanol accumulates up to 8–10% in the fermented mash. The fermented mash is then distilled, fractionated and rectified after the removal of yeast sludge (Pathade, 2003). The residue of the fermented mash which comes out as liquid waste is termed as distillery slop or spentwash (Pathade, 2003; Singh et al, 2004; Nandy et al, 2002).

In literature, apart from yeasts, a bacterial strain, *Zymomonas mobilis*, has been demonstrated as a potential candidate for ethanol production (Chandraraj and Gunasekaran, 2004).

2.2.3 Distillation

Distillation is a two-stage process and is typically carried out in a series of bubble cap fractionating columns. The first stage consists of the distillation column and is followed by rectification columns. The cell free fermentation broth is preheated to about $90^{O}C$ by heat exchange with the effluent (‘‘slop’’) and then sent to the degasifying section of the distillation column. Here, the liquor is heated by live steam and fractionated to give about 50% alcohol. The wastewater discharge from the distillation column is the slop. The alcohol vapors are led to the rectification column where by reflux action, 95 -97% alcohol is tapped, cooled and collected. The condensed water from this stage, known as ‘‘spentlees’’ is usually pumped back to the distillation column.

2.2.4 Packaging

Rectified spirit (95-97% ethanol by volume) is marketed directly for the manufacture of chemicals such as acetic acid, acetone, oxalic acid and absolute alcohol. Denatured ethanol for industrial and laboratory uses typically contains 60–95% ethanol as well as between 1% to 5% each of methanol, isopropanol, methyl isobutyl ketone (MIBK), ethyl acetate, etc. (Skerratt, 2004).

For beverages, alcohol is matured and blended with malt alcohol and diluted to the requisite strength. Then it is bottled appropriately in a bottling plant.

Anhydrous ethanol for fuel-blending applications (‘‘power alcohol’’) requires concentration of ethanol to 99.5% purity.

2.3 Molasses-based distillery wastewaters generation and characteristics

Alcohol production from molasses generates large volumes of high strength wastewater that is of serious environmental concern. The aqueous effluent stream from distilleries known as sugarcane molasses wastewater is approximately 12–15 times the volume of the produced alcohol. The wastewater from distillery is characterized by extremely high chemical oxygen demand (COD) (80,000–100,000 mg/l) and biochemical oxygen demand (BOD) (40,000–50,000 mg/l). However, the amount and the characteristics of the sugarcane molasses wastewater are highly variable and dependent on the raw material used and on the ethanol production process (Pant and Adholeya, 2007; Satyawali and Balakrishanan, 2008). Washing water used to clean the fermenters, cooling water and boiler water further contribute to its variability (Pant and Adholeya, 2007). The main source of wastewater is the distillation step wherein large volumes of dark brown effluent (termed as spentwash, stillage, slop or vinasse) is generated with a temperature range of 70–80 °C, acidic (low pH), and with high concentration of organic materials and solids (Yeoh, 1997; Nandy et al., 2002).

Apart from high organic content, distillery wastewater also contains nutrients in the form of nitrogen, phosphorus and potassium (Mahimairaja and Bolan, 2004) that can lead to eutrophication of water bodies. Further, its dark color leads to widespread damage to aquatic life. Table 3 summarizes the typical characteristics of distillery slop generated in Thai distilleries using molasses.

Table 3 Quantities and characteristics of distillery slop generated in 32 Thai distilleries (The Excise Department, 1983).

Parameters	Values	
	Range	Average
Discharge volume (m^3/ day)	23 - 400	90
pH	2.3 – 5.5	3.7
Temperature (°C)	53 – 100	88.6
BOD_5 (mg/l)	17,500 – 45,000	27,475
COD (mg/l)	56,970 – 193,600	118,100
COD/BOD_5	1.90 – 7.67	4.3
Suspended solid (SS) (mg/l)	5,240 – 23,830	11,319
Total solid (TS) (mg/l)	36,280 – 123,640	75,830
Total volatile solid (TVS) (mg/l)	30,280 – 59,220	58,520

Table 3 Quantities and characteristics of distillery slop generated in 32 Thai distilleries (The Excise Department, 1983). (continued)

Parameters	Values	
	Range	Average
Total nitrogen (mg/l)	40 – 2,160	940
Phosphate (mg/l)	24 – 380	115
Potassium (mg/l)	2,300 – 8,900	4,760
Sulfate (mg/l)	1,820 – 5,160	3,720

Source: Thailand Institute of Scientific and Technological Research (TISTR)

The recalcitrant nature of wastewater from sugarcane molasses is due to the presence of the dark brown colorants, which are biopolymeric colloidal materials that are negatively charged. Except caramel ,all colorants contain phenolic groups which contribute to their formation. Infrared spectra of alkaline degradative products indicate the presence of high molecular weight amino acids. It has been suggested that most of the phenolic colorants are derived from benzoic and cinnamic acid that are precursors of flavanoids, the yellow pigments of the plants, responsible for color formation. The phenolic acids which form colored complexes with iron or get oxidized to polymeric colorants are o-hydroxy or o-dihydroxy acids (Mane et al., 2006). During heat treatment, Maillard reaction takes place resulting in formation of melanoidins, one of the final products of the Maillard reaction (Pant, 2007; Singh et al., 2004; Mohana et al., 2007; Kumar et al., 1997). Apart from melanoidins, the other recalcitrant compounds present in the waste are caramel, different products of sugar decomposition, anthocyanins, tannins and different xenobiotic compounds (Pandey et al., 2003). The unpleasant odor of the effluent is due to the presence of skatole, indole and other sulfur compounds, which are not decomposed by yeast during distillation (Sharma et al., 2007).

2.4 Melanoidins

Melanoidins are dark brown to black colored natural condensation products of sugars and amino acids, they are produced by non-enzymatic browning reactions known as Maillard reactions (Plavsic et al., 2006). Naturally melanoidins are widely distributed in food (Painter, 1998), drinks and widely discharged in huge amount by various agro-based industries especially from distilleries using sugarcane molasses and fermentation industries as environmental pollutants (Kumar and Chandra, 2006;

Gagosian and Lee, 1981). The structure of melanoidins is still not completely understood but it is assumed that it does not have a definite structure as its elemental composition and chemical structures largely depend on the nature and molar concentration of parent reacting compounds and reaction conditions as pH, temperature, heating time and solvent system used (Ikan et al., 1990; Yaylayan and Kaminsky, 1998).

Food and drinks such as bakery products, coffee and beer having brown colored melanoidins exhibited antioxidant, antiallergenic, antimicrobial and cytotoxic properties as *in vitro* studies have revealed that products from Maillard reaction may offer substantial health promoting effects. They can act as reducing agents, metal chelators and radical scavengers(Borrelli et al., 2003; Plavsic et al., 2006). Besides, these health-promoting properties, melanoidins also have antioxidant properties, which render them toxic to many microorganisms such as those typically present in wastewater treatment systems (Kumar et al., 1997). The resistance of melanoidins to degradation is apparent from the fact that these compounds escape various stages of wastewater treatment plants and finally enters into the environment.

2.4.1 Melanoidin formation pathway

The formation of melanoidins is the result of polymerization reactions of highly reactive intermediates formed during Maillard reaction. A wide range of reactions takes place, including cyclizations, dehydrations, retroaldolizations, rearrangements, isomerizations and further condensations, which lead to the formation of brown nitrogenous polymers and copolymers, known as melanoidins. The molecular weight of colored compounds increases as browning proceeds.

The complexity of Maillard reaction has been extensively studied during recent years and new important pathways and key intermediates have been established (Martins et al., 2001). A scheme of Maillard reaction is shown in Figure 2. Melanoidins are recognized as being acidic compounds with charged nature. With increasing reaction time and temperature, the total carbon content increases, thus promoting the unsaturation of the molecules. The color intensity increases with the polymerization degree. The degree of browning, usually measured via absorbance at 420 nm, is often used to follow the extent of Maillard reaction.

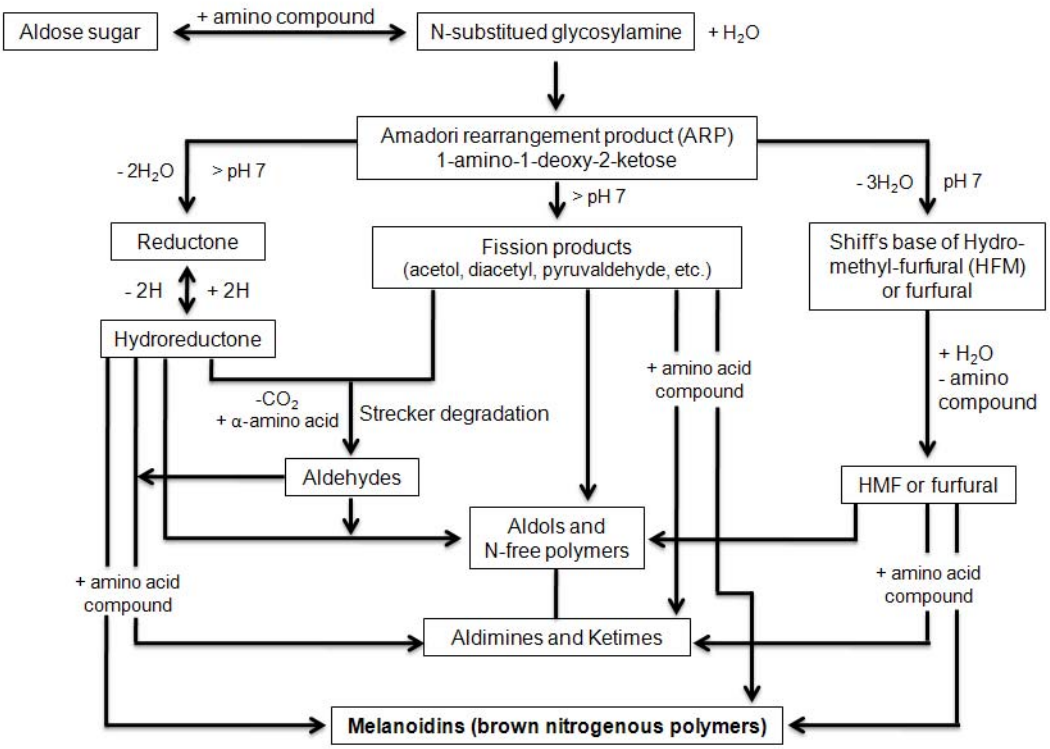

Figure 2 Scheme of Maillard reaction (Martins et al., 2001).

Hayase et al. (1982) reported the formation of a C_3 sugar fragment in early stages of browning reaction between sugar and amines or amino acids, which was identified as methylglyoxal dialkylamine. Fay and Brevard (2004) studied the initial steps of Maillard reaction and reported that the first stable intermediate compound produced in the initial stages of Maillard reaction were Amadori compounds, *N*-substituted 1-amino-1-deoxyketoses, representing an important class of Maillard intermediates, which were produced during the initial phases of Maillard reaction by Amadori rearrangement of corresponding *N*-glycosyl amines.

This type of rearrangement was named after Mario Amadori who was the first to demonstrate the condensation of D-glucose with an aromatic amine. This reaction would yield two structurally different isomers, *N*-substituted glycosyl-amine, which was more labile than the other, N-substituted 1-amino-1-deoxy-2-ketose, towards hydrolysis. Hence, these intermediates of Maillard reaction were termed as Amadori compounds. It has been suggested that marine humic and fulvic acids are formed by

the condensation of sugars with amino acids or proteins via Maillard reaction. Further, the results indicate that various heterocyclic moieties are the main building blocks of humic substances rather than aromatic benzenoid structures (Ikan et al., 1992). Hayashi and Namiki (1986) have also observed that C_3 imine formation followed the pattern of C_2 imine formation, and was well correlated to decrease in the amount of glucosylamine and an increase in the formation of Amadori products. Reaction of Amadori products with n-butylamine rapidly produced C_3 compound in a manner similar to that of glucose-*n*-butylamine system. These results indicated the possibility of participation of Amadori products in the formation of C_3 compound. In spite of large research work done on the Maillard reaction, many parts as mechanism of melanoidins formation at later final stages of Maillard reaction are still obscure. However, the proposed mechanisms reviewed above present a clear picture of melanoidins formation through Maillard amino–carbonyl reaction.

2.4.2 Structure of melanoidin polymer

The elucidation of the chemical structure of melanoidins is difficult due to the complexity of the Maillard reaction. Kato and Tsuchida (1981) proposed a major repeating unit for melanoidins prepared from glucose and butylamine (pH 5.0–6.5). The structure is useful for explaining the great increase of the reductone content of melanoidins on heat treatment under anaerobic conditions. However, changing reaction conditions play an important role in the fundamental structure of melanoidins. This means that it cannot be assumed that melanoidins have a regular composition with repeating units. For this reason, Cämmerer and Kroh (1995) proposed a general structure for melanoidins prepared from monosaccharides and glycine. The chemical structure of investigated melanoidins aforementioned above is shown in Figure 3.

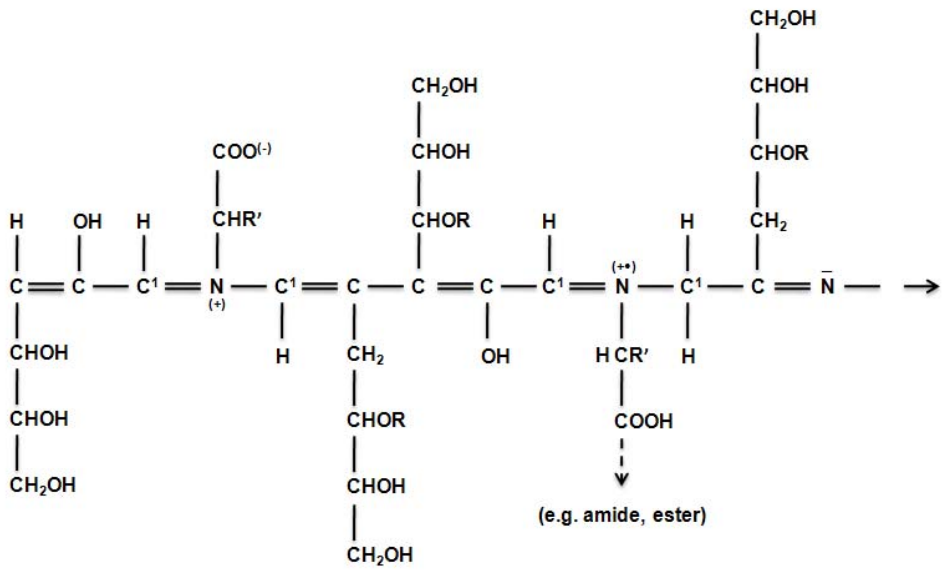

Figure 3 Proposal for the general structure of the melanoidin polymer (Cämmerer and Kroh, 1995). R:H or saccharides. R': side chain of amino acid.

The basic structure is formed by α-dicarbonyl Maillard reaction intermediates, partially branched by amino compounds and with many reactive centers that make possible further decarboxylation and dehydration reactions. The structure of the real melanoidins is likely to be a result of different reactions from the basic framework. Yaylayan and Kaminsky (1998) isolated a brown nitrogen-containing polymer formed in the Maillard mixture. The structure was consistent with the one proposed by Cämmerer and Kroh (1995). This polymer exhibited a strong absorption band at 1607 cm^{-1} in the FTIR spectrum, attributed to extensive conjugation. Pyrolysis of the isolated polymer produced typical Amadori products, such as pyrazines, pyrroles, pyridines and furans. Cämmerer et al., (2002) have recently suggested a new model of a basic skeleton for melanoidins formed from carbohydrates and amino acid (Figure 4)

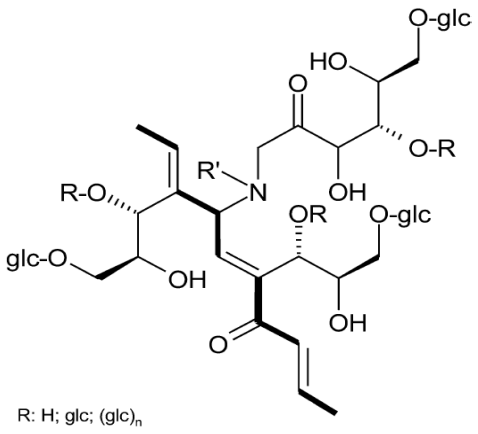

Figure 4 Basic melanoidin structure formed from carbohydrates and amino acid (Cämmerer et al., 2002).

Although the chemical structure of melanoidins is not clearly understood, but some part of the chemical structure of model melanoidins have recently been elucidated by different spectral studies such as ^{1}H NMR, CP-MAS NMR, etc. (Ikan et al., 1990; Ikan et al., 1992; Larter and Douglas, 1980). The chemical investigations have revealed that natural and synthetic melanoidins both have similar elemental (CHON) compositions, spectroscopic properties and electrophoretic mobilities at various pH values (Migo et al., 1997; Ikan et al., 1990; Ikan et al., 1992). However, the nitrogen contents, acidities and electrophoretic behavior of the polymers all reflect functional group distributions inherited from the amino acids (Hedges, 1978).

In spite of these studies, the melanoidins chromophore has not been yet identified. Hence, the chemical structure of the so-called melanoidin is still not clear but probably it does not have a definite one and there exists various types of melanoidins differing in structure depending on parent reactants and reaction conditions as pH, temperature and reaction time. Moreover, it further needs intensive investigations with more refined recent and advanced techniques for the elucidation of chromophore structure to deduce the main skeleton of melanoidin polymer.

2.5 Environmental hazards of molasses-based distillery wastewaters

Sugarcane molasses-based distillery wastewaters disposal into the environment is hazardous and has high pollution potential. High COD, total nitrogen

and total phosphate content of the effluent may result in eutrophication of natural water bodies (Kumar et al., 1997). The highly colored components of the molasses wastewater reduce sunlight penetration in rivers, lakes or lagoons which in turn decrease both photosynthetic activity and dissolved oxygen concentration affecting aquatic life. Kumar et al. (1995) evaluated the toxic effect of distillery effluent on common guppy, *Lesbistes reticulates* and observed remarkable behavioural changes with varying effluent concentration. Kumar and Gopal (2001) reported hematological alterations in fresh water catfish, *Channa punctatus*, exposed to distillery effluents. Saxena and Chauhan (2003) investigated the influence of distillery effluent on oxygen consumption in fresh water fish, *Labeo rohita* and observed that the presence of inorganic and organic salts in the effluent interfered with the respiration in the fish. The coagulation of gill mucous decreased dissolved oxygen consumption causing asphyxiation. Matkar and Gangotri (2003) observed concentration dependent toxicity of distillery effluent on the fresh water crab, *Barythephusa guerini*.

Disposal of sugarcane molasses wastewater on land is equally hazardous to the vegetation. It was reported to reduce soil alkalinity and manganese availability, thus inhibiting seed germination (Kumar et al., 1997). Kannan and Upreti (2008) reported highly toxic effects of raw distillery effluent on the growth and germination of *Vigna radiata* seeds even at low concentration of 5% (v/v). Application of distillery effluent to soil without proper monitoring, perilously affects the groundwater quality by altering its physicochemical properties such as color, pH, electrical conductivity, etc. due to leaching down of the organic and inorganic ions (Jain et al., 2005). Juwarkar and Dutta (1990) evaluated the impact of application of distillery effluent on soil microflora. Irrigation with raw distillery effluent resulted in low overall bacterial and actinomycetes count. Nitrogen fixing bacteria *Rhizobium* and *Azotobacter* reduced considerably. However, population of fungi increased. Anaerobically treated effluent also showed similar reduction as previously mentioned with bacteria but not as much as that of the raw effluent.

2.6. Treatment technologies for sugarcane molasses wastewater

Several technologies have been explored for reducing the pollution load of sugarcane molasses wastewater. Majority of these methods decolorize the effluent by either concentrating the color into the sludge or by breaking down the colored molecules. These treatment technologies are discussed in detail in the following section.

2.6.1 Treatments based on physicochemical methods

2.6.1.1 Adsorption

Among the physicochemical treatment methods, adsorption on activated carbon is widely employed for removal of color and specific organic pollutants. Activated carbon is a well known adsorbent due to its extended surface area, microporous structure, high adsorption capacity and high degree of surface reactivity. Previous studies on decolorization of molasses wastewater include adsorption on commercial as well as indigenously prepared activated carbons (Satyawali and Belakrishanan, 2008). Decolorization of synthetic melanoidins using commercially available activated carbon as well as activated carbon produced from sugarcane bagasse was investigated (Satyawali and Balakrishnan, 2007). The adsorptive capacity of the different activated carbons was found to be quite comparable.

Pendyal et al. (1999) found twenty-four granular activated carbons (GACs) made from mixtures of four binders (coal tar, sugarcane molasses, sugar beet molasses, and corn syrup) and three agricultural by-products (rice hulls, rice straw, and sugarcane bagasse) which were evaluated for their ability to remove sugar colorants (molasses color removal and sugar decolorization). These properties were compared to those of two commercial reference carbons. GACs made from sugarcane bagasse, in general, possessed the best ability to remove sugar colorants and were closest to the reference carbons in this regard. In fact, the four highest ranked GACs all used bagasse as a feedstock along with four different binders. Therefore, the ability to remove sugar colorants appears to be by-product dependent with the binder playing a minor role.

2.6.1.2. Oxidation processes

Ozone is a powerful oxidant for water and waste water treatment. Once dissolved in water, ozone reacts with a great number of organic compounds in two different ways: by direct oxidation as molecular ozone or by indirect reaction through formation of secondary oxidants like free radical species, in particular the hydroxyl radicals. Both ozone and hydroxyl radicals are strong oxidants and are capable of oxidizing a number of compounds (Bes-Pia 2003).

Oxidation by ozone could achieve 80% decolorization for biologically treated molasses wastewater with simultaneous 15.25% COD reduction. It also resulted in improved biodegradability of the effluent. However, ozone only transforms the chromophore groups but does not degrade the dark colored polymeric compounds in the effluent (Alfafara et al., 2000; Peña et al., 2003). Ozone in combination with UV radiation enhanced molasses wastewater degradation in terms of COD; however,

ozone with hydrogen peroxide showed only marginal reduction even on a very dilute effluent (Beltran et al., 1997). Samples exposed to 2 h ultrasound pre-treatment displayed 44% COD removal after 72 h of aerobic oxidation compared to 25% COD reduction shown by untreated samples.

The Fenton's oxidation technology is based on the production of hydroxyl radicals .OH, which has an extremely high oxidation potential. Fenton's reagent, which involves homogeneous reaction and is environmentally acceptable, is a mixture of hydrogen peroxide and iron salts (Fe^{2+} or Fe^{3+}) which produces hydroxyl radicals which ultimately leads to decolorization of the effluent (Pala and Erden, 2005)

Another option is photo-catalytic oxidation that has been studied using solar radiation and TiO_2 as the photocatalyst (Kulkarni, 1998). Use of TiO_2 was found to be very effective as the destructive oxidation process leads to complete mineralization of effluent to CO_2 and H_2O.

2.6.1.3 Coagulation and flocculation

Coagulation is the destabilization of colloids by neutralizing the forces that keep them apart. Cationic coagulants provide positive electric charges to reduce the negative charge (zeta potential) of the colloids. As a result, the particles collide to form larger particles (flocs). Flocculation is the action of polymers to form bridges between the flocs, and bind the particles into large agglomerates or clumps. Bridging occurs when segments of the polymer chain adsorb on different particles and help particles aggregate. Generally coagulation seems to be an expensive step taking into account expenses of chemicals and sludge disposal (Ecologix Environmental system, LLC, 2008)

2.6.1.4 Membrane treatment

Pre-treatment of molasses wastewater with ceramic membranes prior to anaerobic digestion was reported to halve the COD from 36,000 to 18,000 mg/l (Chang et al., 1994). The total membrane area was 0.2 m^2 and the system was operated at a fluid velocity of 6.08 m/s with 0.5 bar transmembrane pressure.

Electrodialysis has been explored for desalting molasses wastewater using cation and anion exchange membranes resulting in 50–60% reduction in potassium content (de Wilde, 1987). Vlyssides et al. (1997) reported the treatment of vinasse from beet molasses by electro dialysis using a stainless steel cathode, titanium alloy anode and 4% w/v NaCl as electrolytic agent. Up to 88% COD reduction at pH 9.5

was obtained. However, the COD removal percentage decreased at higher wastewater feeding rates.

In a recent study, Nataraj et al. (2006) reported pilot trials on distillery spent wash using a hybrid nanofiltration (NF) and reverse osmosis (RO) process. NF was primarily effective in removing the color and colloidal particles accompanied by 80 and 45% reduction in total dissolved solids and chloride concentration, respectively, at an optimum feed pressure of 30–50 bar.

2.6.1.5 Evaporation and combustion

Molasses wastewater containing 4% solids can be concentrated to a maximum of 40% solids in a quintuple-effect evaporation system with thermal vapor recompression (Bhandari et al., 2004; Gulati, 2004). The condensate with a COD of 280 mg/l can be used in fermenters. The concentrated mother liquor is spray dried using hot air at 180^{O}C to obtain a desiccated powder. The powder is typically mixed with 20% agricultural waste and burnt in boiler. Combustion is also an effective method of on-site vinasse disposal as it is accompanied by production of potassium-rich ash that can be used for land application (Cortez and Perèz, 1997).

2.6.1.6 Other treatments

Pikaev (2001) applied radiation technology for treatment of distillery waste. The study involved a combined treatment of electron beam and coagulation using $Fe_2(SO)_3$ which resulted in a decrease in optical absorption in the UV region by 65–70% in the treated effluent. Ultrasound technology was also applied for the treatment of distillery effluent. Studies were carried out to find out the efficacy of the ultrasonic irradiation as a pretreatment step and the results indicated that ultrasound treatment enhanced the biodegradability of the distillery waste water (Sangave and Pandit, 2004). Chaudhari et al. (2008) proposed a novel catalytic thermal pretreatment or catalytic thermolysis to recover the majority of its energy content with consequent COD and BOD removal. This process resulted in the formation of settleable solid residue and the slurry obtained after the thermolysis exhibited very good filtration. It can be used as a fuel in the combustion furnaces and the ash obtained can be blended with organic manure and used in agriculture/horticulture.

Various physicochemical methods such as adsorption, coagulation–flocculation, and oxidation processes like Fenton's oxidation, ozonation, electrochemical oxidation using various electrodes and electrolytes, nanofiltration, reverse osmosis, ultrasound and different combinations of these methods have also been tested for the treatment of distillery effluent. As mentioned above, sugarcane

molasses wastewaters have been reported to be decolorized by various physiochemical methods which are summarized in Table 4 below.

Table 4 Summary of various physicochemical treatments used for the treatment of sugarcane molasses-based distillery wastewaters and their efficiency

Treatment	**COD removal (%)**	**Color removal (%)**	**References**
Adsorption			
Chitosan, a biopolymer was used as anion exchanger	99	98	Lalvo et al., 2000
Chemically modified bagasse			Mane et al., 2006
DEAE bagasse	40	51	
CHPTAC bagasse	25	50	
Activated carbon prepared from agro industrial waste			Satyawali and Balakrishanan, 2008
Phosphoric acid carbonized bagasse	23	50	
Commercially available activated carbon			
AC (ME)	76	93	
AC (LB)	88	95	
Coagulation–flocculation			
Flocculation of synthetic melanoidins was carried out by various inorganic ions			Migo et al., 1997
Polyferric hydroxysulphate (PFS)	NR	95	
Ferric chloride ($FeCl_3$)	NR	96	
Ferric sulphate ($Fe_2(SO_4)_3$)	NR	95	
Aluminium sulphate ($Al_2(SO_4)_3$)	NR	83	
Calcium oxide (CaO)	NR	77	
Calcium chloride ($CaCl_2$)	NR	46	

Table 4 Summary of various physicochemical treatments used for the treatment of sugarcane molasses-based distillery wastewaters and their efficiency**.** (Continued)

Treatment	COD removal (%)	Color removal (%)	References
Oxidation processes			
Fenton's oxidation	88	99	Pala and Erden, 2005
Ozonation	15–25	80	Pena et al., 2003
Electrochemical oxidation			
Graphite electrodes	80.6	95.6	Manisankar et al., 2003
Lead dioxide coated on titanium	90.8	98.5	
Ruthenium dioxide coated on titanium	92.1	99.5	
Electrocoagulation and electro Fenton	92.6	–	Yavuz, 2007
Membrane technologies			
Reverse osmosis	99.9	–	Nataraj et al., 2006
Nanofiltration	97.1	100	

NR: Not reported.

Physicochemical treatment methods are effective in both color and COD removal. Nevertheless the drawbacks associated with these methods are excess use of chemicals, sludge generation with subsequent disposal problems, high operational costs and sensitivity to variable water input. Considering the advantages and the disadvantages of different treatment technologies, no single technology can be used for complete treatment of molasses wastewater. Hence, there is a need to establish a comprehensive treatment approach involving several technologies sequentially.

2.6.2 Treatments based on biological methods

Biological treatment of molasses wastewater is either aerobic or anaerobic but in most cases a combination of both is used. Anaerobic treatment is an accepted practice and various high rate reactor designs have been tried at pilot and full scale operation. Aerobic treatment of anaerobically treated effluent using different microbial populations has also been explored. Majority of biological treatment technologies remove color by either concentrating the color into sludge or by partial or complete breakdown of the color molecules. These methods are discussed in detail in the following section.

2.6.2.1 Anaerobic systems

The high organic content of molasses wastewater makes anaerobic treatment attractive in comparison to direct aerobic treatment. Anaerobic digestion is viewed as a complex ecosystem in which physiologically diverse groups of microorganisms operate and interact with each other in a symbiotic, synergistic, competitive or antagonistic association. In the process methane and carbon dioxide are generated (Jain et al., 1990). Molasses wastewater treatment using anaerobic process is a very promising re-emerging technology which presents interesting advantages as compared to classical aerobic treatment. It produces very little sludge, requires less energy and can be successfully operated at high organic loading rates; also, the biogas thus generated can be utilized for steam generation in the boilers thereby meeting the energy demands of the unit (Nandy et al., 2002). Further, low nutrient requirements and stabilized sludge production are other associated benefits (Jiménez et al., 2004). However, the performance and treatment efficiency of anaerobic process can be influenced both by inoculum source and feed pre-treatment. These processes have been sensitive to organic shock loadings, low pH and showed slow growth rate of anaerobic microbes resulting in longer hydraulic retention times (HRT). This often results in poor performance of conventional mixed reactors. In order to solve these problems, several high rate configurations have been developed for treating soluble wastewater at relatively shorter HRTs (Patel and Madamwar, 2000).

Anaerobic lagoon

Anaerobic lagoons are the simplest choice for anaerobic treatment of molasses wastewater. Rao (1972) carried out the pioneering research work in the field of distillery waste management by employing two anaerobic lagoons in series,

resulting in BOD removal ranging from 82 to 92%. However, the lagoon systems are seldom operational, souring being a frequent phenomenon. Large area requirement, odor problem and chances of ground water pollution are drawbacks (Singh et al., 2004).

Conventional anaerobic systems

The conventional digesters such as continuous stirred tank reactors (CSTR) are the simplest form of closed reactors with gas collection. Treatment of molasses wastewater in CSTR has been reported in single as well as biphasic operations, resulting in 80–90% COD reduction within a period of 10–15 days (Pathade, 2003). The HRT in CSTR-type reactor is determined by the specific growth rate of the slowest growing microorganism in the system. This generally means that very high HRT values are required to achieve an acceptable level of degradation. The high HRT values make the CSTR concept less feasible and unattractive for treatment of the wastewaters (Kleerebezem and Macarie, 2003).

Anaerobic batch reactors

Treatment of distillery waste using batch reactors has not been widely attempted. Treatment of winery wastewater was investigated using an anaerobic sequencing batch reactor (ASBR). The reactor was operated at an OLR of 8.6 kg COD m^{-3} d^{-1} with soluble COD removal efficiency greater than 98% with HRT of 2.2 days (Ruiz et al., 2002).

Banerjee and Biswas (2004) designed a semi-continuous batch digester to investigate biomethanation of distillery waste in mesophilic and thermophilic range of temperatures. The study revealed that there was an important effect of the temperature of digestion and of substrate concentration in terms of BOD and COD loading on the yield of biogas as well as its methane content. Maximum BOD reduction (86.01%), total gas production and methane production (73.23%) occurred at a BOD loading rate of 2.74 kg m^{-3} at 50 °C digestion temperature.

Anaerobic fixed film reactors

In fixed film reactors, the reactor has a biofilm support structure (media) for biomass attachment. Figure 5 shows the schematic representation of an anaerobic fixed film reactor. Fixed film reactor offers the advantages of simplicity of construction, elimination of mechanical mixing, better stability even at higher loading rates and capability to withstand toxic shock loads. The reactors can recover very

quickly after a period of starvation (Rajeshwari et al, 2000). Amongst numerous anaerobic reactors developed for biomethanation, anaerobic fixed film reactors (AFFR) have emerged as the most popular one compared to other reactors due to availability of large biomass in the reactor (Patel and Madamwar, 2002). The nature of the media used for biofilm attachment has a significant effect on reactor performance. A wide variety of materials like glass bead, red drain clay, sand and a number of different plastics and porous materials such as needle punched polyesters, polyureathane foam and sintered glass (Perez, 1997), waste tire rubber (Borja, 1996), poly(acrylonitrile–acrylamide) (Lalov, 2001), corrugated plastic (Perez-Garcia, 2005), etc., have been used as non-porous support media at laboratory as well as pilot-scale

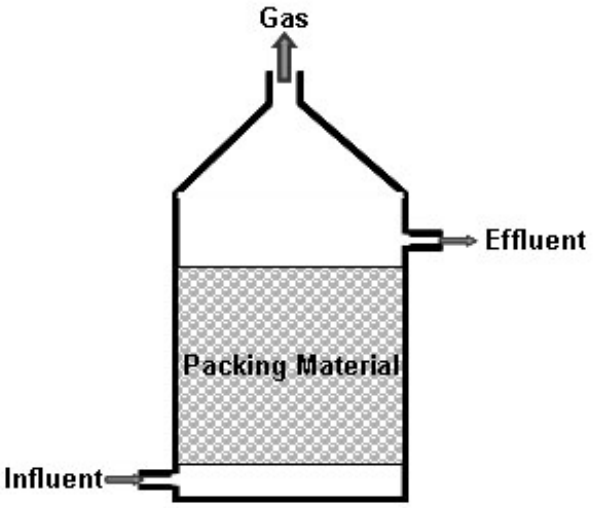

Figure 5. Schematic diagram of anaerobic fixed film reactor (modified from Kansal et al., 1998).

In another study, Perez-Garcia et al., (2005) studied the influent pH conditions in fixed film reactors for anaerobic thermophilic treatment of wine distillery wastewaters. They showed that the pH of the influent influenced the performance of the biodegradation process and the depurative efficiency was higher with alkaline influent. The operation with acidic influent allowed the reactor to operate at OLR around 5.6 kg COD m^{-3} d^{-1} (HRT: 1.5 days), maintaining total COD removals of 77.2%; the operation with alkaline influent allowed total COD removals of 76.8% working at OLR around 10.5 kg COD m^{-3} d^{-1}. The greatest efficiency of substrate removal was 87.5% for OLR 3.2 kg COD m^{-3} d^{-1} and HRT of 4 days operating with alkaline influent. Therefore, the operation with alkaline influent implicates higher levels of purifying efficiency for similar organic load rate.

Acharya et al., (2008) performed a comparative study of low cost packing materials for the treatment of distillery spent wash using anaerobic fixed film reactors. Coconut coir was found to be the best supporting material, as the system supported the treatment at very high organic loading rate of 31 kg COD m^{-3} d^{-1} with 50% COD reduction. Charcoal and Nylon fibers were other packing materials used in the study. Charcoal was able to retain the active biomass at the OLR of 15.5 kg COD m^{-3} d^{-1} resulting in more than 60% COD reduction whereas nylon fibers failed to support the biofilm development even at higher HRT and lower OLR.

Upflow anaerobic sludge blanket (UASB) reactors

The UASB process has been successfully used for the treatment of various types of wastewaters (Lettingar and Holshoff Pol, 1991). UASB reactor systems belong to the category of high rate anaerobic wastewater treatment and hence it is one of the most popular and extensively used reactor designs for treatment of distillery wastewaters globally. The success of UASB depends on the formation of active and settleable granules (Fang et al., 1994). These granules consist of aggregation of anaerobic bacteria, self immobilized into compact forms. This enhances the settelability of biomass and leads to an effective retention of bacteria in the reactor (Akunna and Clark, 2000). Particularly attractive features of the UASB reactor design include its independence from mechanical mixing, recycling of sludge biomass (Kalyazhnyi et al., 1997) and ability to cope up with perturbances caused by high loading rates and temperature fluctuations (Sharma and Singh, 2002). The schematic representation of an UASB reactor is shown in Figure 6. The UASB technology is well suited for high strength distillery wastewaters when the process has been successfully started up and is in stable operation.

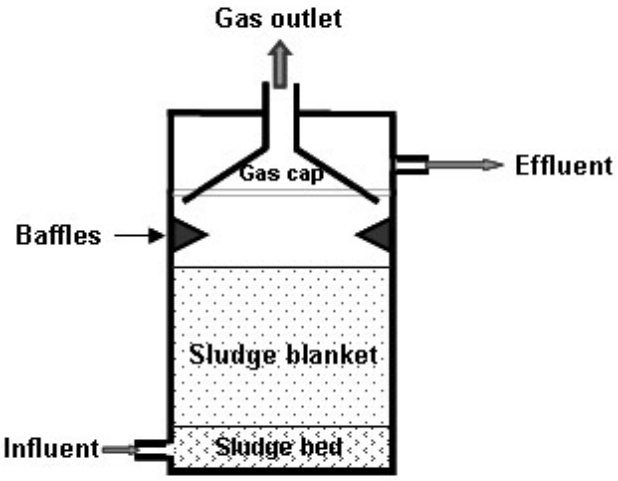

Figure 6 Schematic diagram of anaerobic UASB reactor (modified from Kansal et al., 1998).

Potable of malt whisky distillery, a liquid waste product from the malt whisky industry, was treated in a laboratory scale UASB reactor, pH control was of interest to attain a high COD reduction (Goodwin and Stuart, 1994). There is normally a rise in the pH due to ammonia production during the process of digestion. The maximum loading rate for a stable operation was 15 kg COD m^{-3} d^{-1} at a retention time of 2.1 days.

Successful operation of the UASB reactors for treating distillery waste at psychrophilic temperatures (4–10 °C) was also studied by operating one and two-stage UASB reactors. The organic loading rate varied from 4.7 to 1.3 g COD at HRT of 6–7 days for one-stage reactor and 2 days for the two-stage reactor. The average total COD removal for vinasses waste waters was 60% in the one-stage reactor and 70% in the two-stage reactor. Therefore, application of high recycle ratios is essential for enhancement of UASB pretreatment under psychrophilic conditions (Kalyazhnyi et al., 2001).

Uzal et al., (2003) investigated an anaerobic treatment of whisky distillery waste in two-stage UASB reactors and concluded that the system worked efficiently even at OLRs as high as 39 kg COD m^{-3} d^{-1} resulting in 95–96% COD reduction.

Anaerobic fluidized bed reactors

In anaerobic fluidized bed reactor (AFB), the medium which support bacteria attachment and growth is kept in the fluid state by drag forces exerted by the up flowing wastewater. The media used are small particle size sand, activated carbon, etc. In the fluidized state, each medium provides a large surface area for biofilm formation and growth. It enables the attainment of high reactor biomass hold-up and promotes system efficiency and stably. The schematic representation of an anaerobic fluidized bed reactor is shown in Figure 7.

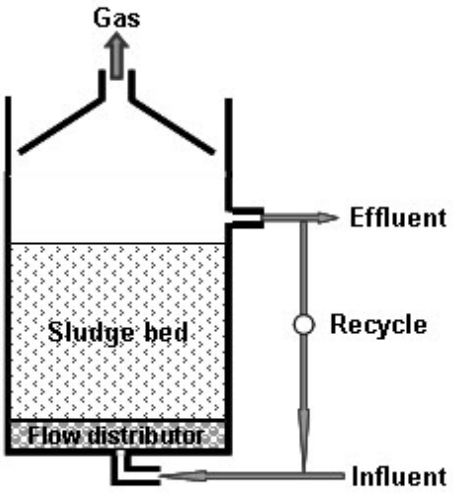

Figure 7 Schematic diagram of anaerobic fluidized bed reactor (modified from Kansal et al., 1998).

Kida et al., (1995) studied the biological treatment of Shochu distillery wastewater using an anaerobic fluidized bed reactor. The maximum loading rate of 22 kg TOC m^{-3} d^{-1} could be achieved by the addition of nickel, cobalt and diluting the waste. This resulted in 70% TOC reduction.

Ability of anaerobic fluidized bed reactor to treat high strength wastewaters like distillery waste under thermophilic temperatures was studied by Perez et al., (1997). The results showed that AFB systems can achieve over 82.5% COD reduction at a COD loading rate of 32.3 kg COD m^{-3} d^{-1} corresponding to HRT of 0.46 day. The highest efficiency of substrate removal was 97% for an organic loading rate of 5.9 kg COD m^{-3} d^{-1} and HRT of 2.5 days.

2.6.2.2 Aerobic systems

Anaerobically treated distillery wastewater still contains high concentrations of organic pollutants and then cannot be discharged directly. The partially treated spent wash has high BOD, COD and suspended solids. It can reduce the availability of essential mineral nutrients by trapping them into immobilized organic forms, and may produce phytotoxic substances during decomposition. Stringent regulations on discharge of colored effluent impede direct discharge of anaerobically treated effluent (Nandy et al., 2002). Therefore, aerobic treatment of sugarcane molasses wastewater has been mainly attempted for the decolorization of the major colorant, melanoidins, and for reduction of the COD and BOD. A large number of microorganisms such as bacteria (pure and mixed culture), cyanobacteria, yeast and fungi have been isolated in recent years and are capable of degrading melanoidins and thus decolorizing the molasses wastewater. The aerobic methods have been described below.

Activated sludge process

The most common wastewater treatment is the activated sludge process wherein research efforts are targeted at improvements in the reactor configuration and performance. For instance, aerobic sequencing batch reactor (SBR) was reported to be a promising solution for the treatment of effluents originating from small wineries (Torrijos and Moletta, 1997). The treatment system consisted of a primary settling tank, an intermediate retention trough, two storage tanks and an aerobic treatment tank. A start up period of 7 days was given to the aerobic reactor and the system resulted in 93% COD and 97.5% BOD removal. The activated sludge process and its variations utilize mixed cultures. To enhance the efficiency of aerobics systems, several workers have focused on the treatment by pure cultures.

Though aerobic treatment like the conventional activated sludge process is presently practiced by various molasses-based distilleries and leads to significant reduction in COD, the process is energy demanding and the color removal is still unsatisfactory. Thus several pure cultures of fungi, bacteria and algae have been investigated specifically for their ability to decolorize the sugarcane molasses wastewater as discussed earlier. Pure bacterial and fungal cultures have been studied to develop bioprocess for melanoidins decolorization of molasses wastewater. However, the performance of fungal decolorization was limited by long growth cycle and moderate decolorization rate. In contrast, the bacterial decolorization is normally faster, but it may require a mixed community to decolorize melanoidins though a combined metabolic mode of individual culture. The bacterial

consortium seems to be more competent for molasses wastewater treatment due to maintenance of microorganism and co-metabolism to enhance the efficiency of melanoidins decolorization. In all instances, it is found necessary to supplement with additional nutrients as well as diluting the effluent for obtaining optimal microbial activity and eventually optimal results. Consequently there is a need to explore more efficient microbes that can decolorize the effluent using it as the sole source of nutrients without much dilution. In addition, these studies are mostly limited to laboratory scale investigations and no pilot/commercial scale operations are reported as yet.

2.6.2.3 Biocomposting process

Biocomposting is a method of activated bioconversion through the aerobic pathway, whereby heterotrophic microorganisms act on carbonaceous materials depending on the availability of the organic source and the presence of inorganic materials essential for their growth. Composting is particularly effective in converting the wet materials to a usable form thereby stabilizing the organic materials and destroying the pathogenic organisms in addition to significant drying of the wet substrates. In the composting process, under aerobic conditions, thermophilic biodegradation of organic wastes at 40-60% moisture content occurs to form relatively stable, humus-like materials (Kannan and Upreti, 2008).

2.6.2.4 Phytoremediation

Phytoremediation of effluents is an emerging low cost technique for removal of toxicants including metals from industrial effluents and is still in an experimental stage. Aquatic plants have excellent capacity to reduce the level of toxic metals, BOD and total solids from the wastewaters (Kumar and Chandra, 2004). Billore et al., (2001) carried out the treatment of distillery effluent in a constructed wetland which comprised of four cells. After a pretreatment in the two first cells the effluent was channeled to cells three and four which contained plants *Typha latipholia* and *Phragmites karka*. This treatment eventually led to 64% COD, 85% BOD, 42% total solids and 79% phosphorus content reduction.

Kumar and Chandra (2004) successfully treated distillery effluent in a two-stage process involving transformation of recalcitrant coloring components of the effluent by a bacterium *Bacillus thuringienesis* followed by subsequent reduction of remaining load of pollutants by a macrophyte *Spirodela polyrrhiza*. A similar biphasic

treatment of the effluent was carried out in a constructed wetland with *Bacillus thuringienesis* and *Typha angustata* by Chandra et al. (2006) which resulted in 98–99% BOD, COD and color reduction after 7 days.

2.6.2.5 Cyanobacterial and algal systems

Cyanobacteria are considered ideal for treatment of molasses wastewater as they, apart from degrading the polymers, also oxygenate waterbodies, thus reduce the BOD and COD levels. Marine *cyanobacteria* such as *Oscillatoria boryna* have also been reported to degrade melanoidins due to the production of H_2O_2, hydroxyl, per hydroxyl and active oxygen radicals, resulting in the decolorization of the effluent (Kalavathi et al., 2001). Patel et al. (2001) have reported 96%, 81% and 26% decolorization of distillery effluent through bioflocculation by *Oscillatoria* sp*., Lyngbya* sp. and *Synechocystis* sp., respectively.

Valderrama et al., (2002) studied the feasibility of combining microalgae, *Chlorella vulgaris* and macrophyte *Lemna minuscule* for bioremediation of wastewater from ethanol producing units. This combination resulted in 61% COD reduction and 52% color reduction. First, the microalgal treatment led to removal of organic matter and further treatment with macrophytes removed other organic matter, color and precipitated the microalgae.

2.6.2.6 Fungal systems

There is a good number of reports showing the role of fungi in decolorization of melanoidins by adsorption to mycelia as well as the role of ligninolytic enzyme (Raghukumar and Rivonkar, 2001; Vahabzadeh et al., 2004; Watanabe et al., 1982). However, the long growth cycle and spore formation limit the performance of the fungal system

Increasing attention has been directed towards utilizing microbial activity for decolorization of molasses wastewater. Several reports have indicated that some fungi in particular have such a potential (Kumar et al., 1998). One of the most studied fungus having ability to degrade and decolorize distillery effluent is *Aspergillus* such as *Aspergillus fumigatus* G-2-6, *Aspergillus niger*, *Aspergillus niveus*, *Aspergillus fumigatus* $U_{B2}60$ brought about an average of 69–75% decolorization along with 70–90% COD reduction (Ohmomo et al., 1987; Miranda et al., 1996; Jimnez et al., 2003; Shayegan et al., 2004; Angayarkanni et al., 2003; Mohammad et al., 2006).

Treatment of distillery spent wash with ascomycetes group of fungi such as *Penicillium* such as *Penicillium decumbens*, *Penicillium lignorum* resulted in about 50% reduction in color and COD, and 70% phenol removal (Jimnez et al., 2003).

Pant and Adholeya (2007) isolated three cultures of fungi and identified them by molecular methods as *Penicillium pinophilum* TERI DB1, *Alternaria gaisen* TERI DB6 and *Pleurotus florida* EM 1303. These cultures were found to produce ligninolytic enzymes and decolorized the effluent up to 50%, 47% and 86%, respectively.

Sirianuntapiboon et al., 2004 isolated 205 yeast strains from Thai-fruit samples and screened. Isolate No. WR-43-6 showed the highest decolorization (68.91%) when cultivated at 30 °C for 8 days in a molasses solution containing 2.0% glucose, 0.1% sodium nitrate, and 0.1% KH_2PO_4, the pH being adjusted to 6.0. This potent strain was identified as *Citeromyces* sp. and showed highest removal efficiencies on stillage from an alcohol distillery (U-MWW). The color intensity, chemical oxygen demand (COD) and biochemical oxygen demand (BOD) removal efficiencies were 75%, almost 100 and 76%, respectively. In a periodical feeding system, *Citeromyces* sp. WR-43-6 showed an almost constant decolorization of 60–70% over 8 day feeding of 10% fresh medium. In a replacement culture system, *Citeromyces* sp. WR-43-6 also gave a constant decolorization (about 75%) during four times replacement.

White rot fungi is another group of widely exploited microorganisms in bioremediation of distillery effluent. White rot fungi produce various isoforms of extracellular oxidases including laccases, manganese peroxidases and lignin peroxidases, which are involved in the degradation of lignin in their natural lignocellulosic substrate. This ligninolytic system of white rot fungi is directly involved in the degradation of various xenobiotic compounds and dyes (Wesenberg et al., 2003). Table 5 gives details on different white rot fungi used in decolorization of distillery effluent and the role of different enzymes in the process.

Miyata et al., 2000 reported a white rot fungus, *Coriolus hirsutus*, exhibited a strong ability to decolorize melanoidins in cultures without supplement of nitrogenous nutrients. Addition of peptone to the cultures lowered the ability of the fungus to decolorize melanoidins, but addition of inorganic nitrogens (Ns), ammonium and nitrate did not bring about any marked reduction in the ability. These results suggested an inhibitory effect of organic nitrogens on melanoidin decolorization. Therefore, for enhancing the decolorization of melanoidins in wastewaters by the fungus, activated sludge pretreatment of the wastewaters was expected to be

effective, *i.e.*, activated sludge is capable of converting available organic nitrogens into inorganic nitrogens. To confirm this, waste sludge heat treatment liquor (HTL), wastewater from a sewage treatment plant was pretreated with activated sludge. In practice, pretreatment of HTL under appropriate conditions accelerated the fungal decolorization of HTL. In the pretreated HTL, the fungus was shown to produce a high level of manganese-independent peroxidase (MIP). Addition of Mn(II) to the pretreated HTL caused a further increase in the decolorization efficiency of the fungus and a marked increase in the manganese peroxidase (MnP) activity. Consequently, the increases in MIP and MnP activities were considered to play an important role in the enhanced ability of *C. hirsutus* to decolorize HTL.

Table 5 Microbial cultures employed for treatment of molasses-based distillery wastewaters.

Culture	Treatment	COD removal	Color removal	Enzymes	References
Fungi					
Coriolus sp. No. 20	Synthetic melanoidins solution was decolorized by the fungus	NR	80%	Sorbose oxidase	Watanebe et al., 1982
Phanerochaete chrysosporium	Free cells as well as Ca alginate immobilized cells decolorized the distillery effluent.	NR	85% (free)	NR	Fahy et al., 1997
		NR	59% (immobilized)	NR	
Trametes versicolor	Anaerobically treated distillery effluent supplemented with sucrose and inorganic N sources was decolorized by the culture in shake flask studies	75%	80%	NR	Benito et al., 1997
Phanerochaete chrysosporium	Both strains decolorized and reduced COD of effluent in presence of (3–5%) glucose and 0.1% yeast extract	73%	53.5%	NR	Kumar et al., 1998
Coriolus versicolor		70%	71.5%	NR	
Coriolus hirsutus	Synthetic as well as wastewater melanoidins were decolorized by the fungus in a medium containing glucose and peptone	NR	80%	MiP and MnP and presence of extracellular H_2O_2	Miyata et al., 1998 and Miyata et al., 2000

Table 5 Microbial cultures employed for treatment of molasses-based distillery wastewaters (continued).

Culture	Treatment	COD removal	Color removal	Enzymes	References
Fungi					
Coriolus hirsutus F044917	The fungal culture was immobilized on PUF and used for decolorization of melanoidins present in heat treated liquor	NR	45%	NR	Fujita et al., 2000
Flavodon flavus	Distillery effluent was decolorized using this marine basidiomycetes in presence of 5% glucose.	NR	80%	Glucose oxidase accompanied with hydrogen peroxide	Rughukumar and Rivonkar, 2001; Rughukumar et al., 2004
Penicillium decumbens	Aerobic/Anaerobic biodegradation of beet molasses wastewater.	50.7%	41%	NR	Jimenez et al., 2003
Coriolus versicolor	The cultures were incubated with cotton stalks in vinasses, media under static conditions. No synthetic carbon or nitrogen sources were used.	49%	63%	NR	Kahraman and Yesilada, 2003
Funalia trogii		62	57		
Phanerochaete chrysosporium		57	37		
Pleurotus pulmonarius		34	43		
Phanerochaete chrysosporium 1557	Effect of Veratryl alcohol and Mn (II) on decolorization of distillery effluent was studied.	NR	75%	LiP and MnP	Vahabzadeh et al., 2004

Table 5 Microbial cultures employed for treatment of molasses-based distillery wastewaters(continued).

Culture	Treatment	COD removal	Color removal	Enzymes	References
Fungi					
Phanerochaete chrysosporium ATCC 24725	The fungus was immobilized on different support materials such as PUF and scouring wet and the decolorization was carried out in a RBC	48%	55%	NR	Guimaraes et al., 2005
P. chrysosporium NCIM 1073	The cultures were employed to study the decolorization of molasses in medium containing 2% glucose under static as well as submerged conditions.	Nil	Nil	NR	Thakkar et al., 2006
P. chrysosporium NCIM 1106		NR	82%	LiP and MnP	
P. chrysosporium NCIM 1197		NR	76%	LiP and MnP	
Marine basidiomycetes NIOCC	Experiments were carried out with 10% (v/v) diluted effluent	NR	100%	Laccase and exoploysaccharide produced by the fungus	D'Souza et al., 2006

Table 5 Microbial cultures employed for treatment of molasses-based distillery wastewaters (continued).

Culture Treatment		COD removal	Color removal	Enzymes	References
Bacteria					
Lactobacillus hilgardii	Decolorization by this bacterial strain when cultivated with melanoidins containing wastewater medium supplemented with 1% of glucose.	NR	28%	NR	Ohmomo et al., 1988
	Decolorization by immobilized cells on calcium alginate.		40%		
Lactobacillus L-2	12.5% diluted wastewater was supplemented with 10 g/l of glucose.	57%	31%	NR	Kumar et al., 1997
Bacillus sp.	The decolorization was studied under anaerobic and thermophilic conditions.	NR	35.5	Decolorization enzyme	Nakajima-Kambe et al., 1999
Aeromonas formicans	Study on predigested distillery effluent.	57%	55%	NR	Jain et al., 2000
Pseudomonas fluorescence	Immobilized cells on porous cellulose carrier.	NR	76%	NR	Dahiya et al., 2001
TA2	Two aerobic bacterial strains isolated from the activated sludge of finally treated distillery effluent were used for the treatment of distillery effluent.	NR	66.67%	NR	Asthana et al., 2001
TA4			63.9%		
Mixed (TA2+TA4)			75%		

Table 5 Microbial cultures employed for treatment of molasses-based distillery wastewaters (continued).

Culture Treatment		COD removal	Color removal	Enzymes	References
Bacteria					
Pseudomonas putida U	Anaerobically treated distillery spent wash in two stage bioreactor (first stage: *Pseudomonas putida*; second stage: *Aeromonas* strain *Ema*)	44.4%	60%	NR	Ghosh et al., 2002
Aeromonas strain Ema		44%	-		
Bacillus cereus	Experiments were carried out with distillery effluent	81%	75%	NR	Jain et al., 2002
Acetogenic bacteria strain No. BP103	Decolorization by the bacterial culture when cultivated in molasses pigments medium containing glucose 3%, yeast extract 0.5%	NR	76%	Sugar oxidase	Sirianuntapiboon et al., 2004b
Mixture of all six isolates: *Pseudomonas, Enterobacter, Stenotrophomonas, Aeromonas, Acinetobacter* and *Klebsiella*	Study on decolorization of molasses spent wash.	44%	NR	NR	Ghosh et al., 2004

Table 5 Microbial cultures employed for treatment of molasses-based distillery wastewaters (continued).

Culture Treatment		COD removal	Color removal	Enzymes	References
Bacteria					
Mixed culture of : *Bacillus thuringiensis* *Bacillus brevis* *Bacillus* sp. (MTCC6506)	The decolorization was studied with 4 types of synthetic melanoidins as follow: GGA (glucose-glutamate-acid) GAA (glucose-aspartic-acid) SGA (sucrose-glutamate-acid) SAA (sucrose-aspartic-acid)	 53.91% 36.13% 63.39% 54.51%	 45.12% 28.88% 50.56% 46.08%	Sugar oxidase and peroxidase	Kumar and Chandra, 2005
Microbacterium hydrocarbonoxydans *Achromobacter xylosoxidans* *Bacillus subtilis* *Bacillus megaterium* *Bacillus anthracis* *Bacillus licheniformis* *Achromobacter xylosoxidans* *Achromobacter* sp. *Bacillus thuringiensis* *Bacillus licheniformis* *Bacillus subtilis* *Staphylococcus epidermidis* *Pseudomonas migulae* *Alcaligens faecalis* *Bacillus cereus*	All the fifteen isolates grown on effluent supplemented medium as a sole carbon source	86.14%	75.5%	NR	Chaturvedi et al., 2006

Table 5 Microbial cultures employed for treatment of molasses-based distillery wastewaters (continued).

Culture Treatment		COD removal	Color removal	Enzymes	References
Bacteria					
Pseudomonas aerugenosa PA01 *Stenotrophomonas maltophila* *Proteus mirobilis*	The decolorization was studied in effluent with low nutrient medium	67%	51%	NR	Mohana et al., 2007
Yeast					
Citeromyces sp. strain no. WR-43-6	Decolorization was observed on stillage from an alcohol distillery (U-MWW).	99.38%	68.91%	NR	Sirianuntapiboon et al., 2004
Cyanobacteria					
Oscillatoria boryana BDU 92181 (marine cyanobacteria)	Decolorization of pure melanoidins (0.1% w/v)	NR	75%	NR	Kalavathi et al., 2001
	Decolorization of crude pigment in distillery effluent (5% v/v)		60%		
Algae					
Mixed culture of Microalgae: *Chlorella vulgaris* Macrophyte: *Lemna minuscule*	Study with diluted wastewater (diluted wastewater from ethanol production to 10% of original concentration)	61%	52%	NR	Valderrama et al., 2002

2.6.2.7 Bacterial systems

Different bacteria capable of both bioremediation and decolorization of molasses wastewater have been isolated. Table 5 gives details on different bacterial isolates employed in decolorization of molasses-based distillery wastewaters. Some of these studies are also discussed in detail in the following section.

Kumar and Viswanathan (1991) isolated bacterial strains from sewage and acclimatized on increasing concentrations of distillery waste, which were able to reduce COD by 80% in 4–5 days without any aeration and the major products left after the degradation process were biomass, carbon dioxide and volatile acids.

Toshiaki et al., 1999 could screen various molasses wastewater-decolorizing microorganisms under thermophilic and anaerobic conditions. Strain MD-32, newly isolated from a soil sample, was selected as the candidate strain. From taxonomical studies, this strain belonged to the genus *Bacillus*, most closely resembling *B. smithii*. The strain decolorized 35.5% of molasses pigment within 20 days at 55°C under anaerobic conditions, but no decolorization activity was observed when cultivated aerobically. At all the concentrations tested, molasses pigment was effectively decolorized by MD-32, with decolorization yields of approximately 15% within 2 days. The molecular weight distribution as determined by gel filtration chromatography revealed that the decolorization of molasses pigment by the isolated strain is accompanied by a decrease in not only small molecules but also large ones.

Acetogenic bacteria strain No.BP103 could decolorize 73.5% of molasses pigments in molasses wastewater supplemented with glucose, yeast extract, and basal mineral salts whereas the decolorization of this strain was decreased to only 9.75% in the absence of supplementary nutrients (Sirianuntapiboon et al., 2004). Nakajima et al. (1999) isolated *Bacillus* sp. which decolorized molasses wastewater up to 35.5% within 20 days at 55 °C under anaerobic conditions. The molecular weight distribution as determination by gel permeation chromatography revealed that there was decrease in color contributing small molecules as well as large molecules.

Jain et al. (2002) isolated three bacterial strains from the activated sludge of a distillery waste water plant identified as *Xanthomonas fragairae*, *Bacillus megaterium* and *Bacillus cereus* which were found to remove COD and color from the distillery effluent in the range of 55–68% and 38–58%, respectively. Two bacterial strains *Pseudomonas putida* U and *Aeromonas* sp. were used to bioremediate anaerobically treated distillery spent wash in a two-stage bioreactor. In the first stage, *P. putida* reduced the COD and color by 44.4% and 60%, respectively. The *Aeromonas* sp., in

the second stage, reduced the COD by 44%. Algal bioassay was used to evaluate the quality of the spent wash before and after treatment. The spent wash was eutrophic before the experimental treatment, but, after treatment, it showed poor algal growth (Ghosh et al., 2002).

Ghosh et al. (2004) also isolated bacterial strains capable of degrading recalcitrant compounds from anaerobically digested spent wash from soil of effluent discharge site which were identified as *Pseudomonas, Enterobacter*, *Stenotrophomonas*, *Aeromonas*, *Acinetobacter* and *Klebsiella* all of which could carry out degradation of PMDE and maximum 44% COD reduction was achieved using these bacterial strains either singly or collectively.

Chaturvedi et al. (2006) isolated and characterized fifteen culturable rhizosphere bacteria of *Phragmites australis* growing in distillery effluent contaminated sites. These fifteen strains were *Microbacterium hydrocarbonoxydans*, *Achromobacter xylosoxidans*, *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus anthracis*, *Bacillus licheniformis*, *Achromobacter xylosoxidans*, *Achromobacter* sp., *Bacillus thuringiensis*, *Bacillus licheniformis*, *Bacillus subtilis*, *Staphylococcus epidermidis*, *Pseudomonas migulae*, *Alcaligens faecalis* and *Bacillus cereus* which collectively brought about 76% decolorization and 85–86% BOD and COD reduction of the effluent within 30 days

Typically, the bacterial decolorization may require a mixed culture to decolorize molasses wastewater through combined metabolic mode of individual bacterial strains. Thus, mixed culture studies have been carried out by several researchers for degradation of different effluents such as textile effluents. As the catabolic activities of microorganisms in a mixed consortium complement each other, obviously the syntrophic interactions present in mixed communities lead to complete mineralization of the effluent (Alkane et al., 2006; Kumar and Chandra, 2006).

Alkane and his co-workers (2006) reported that 69 % decolorization of molasses spent wash was achieved by using soil samples as inoculum instead of isolated microorganisms. Also, Kumar and Chandra (2006) reported that the additional of 1% glucose as a supplementary carbon source was necessary for molasses decolorization by *Bacillus thuringiensis*, *Bacillus brevis*, and *Bacillus* sp. up to 22%, 27.4%, and 27.4% color removal, respectively. The similar pattern was also observed on the decolorization activity of bacterial consortium DMC, comprising of *Pseudomonas aeruginosa* PAO1, *Stenotrophomonas maltophila* and *Proteus mirabilis*, which achieved its maximum molasses decolorization (67%) and 51% COD reduction within 72 h in the presence of 0.5% glucose (Mohana et al., 2007). Hence,

mixed culture studies seem to be more promising for molasses wastewater decolorization.

2.6.3 Membrane bioreactors

A membrane bioreactor (MBR) combines the biological degradation of waste compounds and the physical separation of the biomass and treated water by membrane filtration. MBRs have been introduced over 30 years ago, and until now one of the large industrial applications have been for wastewater treatment e.g., industrial, domestic and municipal (Yang et al., 2006). They have proven quite efficiency in removing both organic and inorganic contaminants as well as biological compounds from wastewater. MBR associate a suspended growth bioreactor and a filtration through porous membrane, which leads to the total retention of biomass (high microbial concentration) and improved biological reactor operation (Lee et al., 2003). Such systems are most often used as replacement for sedimentation i.e., for separation of biomass. Membranes can also be coupled with bioprocesses for wastewater treatment in two ways. Firstly, they can be used to control the transfer of nutrients into bioreactor or to extract pollutants from wastewaters which are untreatable by conventional biological processes i.e., melanoidins. The target pollutants are then removed in a reactor with the suitable environmental conditions for biological treatment. Secondly, they can be used for mass transfer of gases, usually oxygen for aerobic processes (Brindle and Stephenson, 1996).

According to these positive aspects, MBR has been applied to various wastewater treatments and has successfully treated effluents from a range of industrial wastewaters, including textiles, dairy, food, beverage, paper and pulp, metal fabrication, rendering and chemical manufacture. During the last years the application of membrane bioreactors (MBR) to domestic wastewater treatments has noticeably increased (Judd, 2006) and the interest to apply this emerging technology to industrial wastewater has been growing up (Figure 8).

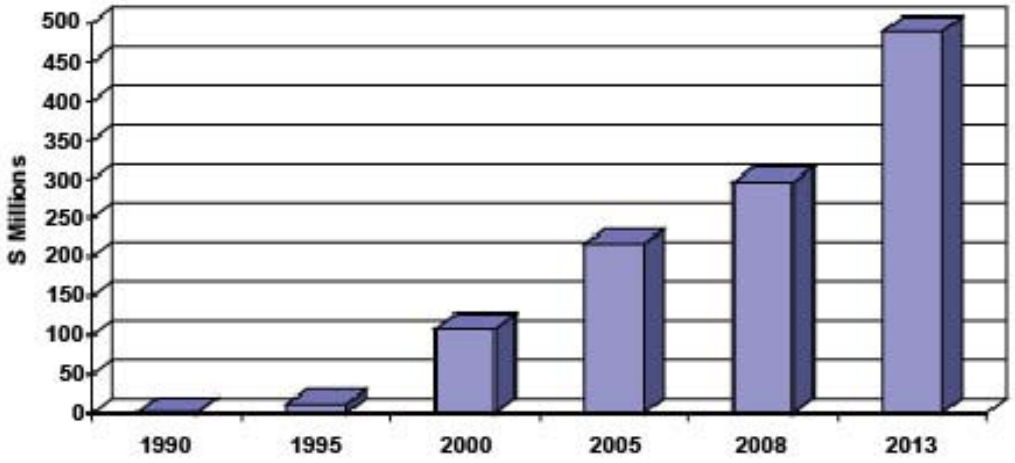

Figure 8 Forecasting the global market value of membrane bioreactor, 1990-2013 (Source: BCC Research)

2.6.3.1 Advantages of membrane bioreactors

There are many advantages in using a MBR process, including superior organics removal, enhanced nutrient removal stability, lower sludge production, smaller footprint, effluent disinfection and high loading rate capabilities (Stephenson et al., 2000). The list of the principle advantages and disadvantages of MBR are shown in Table 6.

Table 6. Advantages and disadvantages of membrane bioreactor.

Advantages	Disadvantages
complete removal of the suspended solids	susceptible to membrane fouling
compact plant size	high capital cost
high rate of degradation	unproven at full-scale, depending on the applications
flexibility in operation	process complexity
low rate of sludge production	
disinfection and odor control	
prolonged microorganisms retention time	
treatment of recalcitrant and toxic pollutants	

2.6.3.2 System configurations

The wide array of membrane types allow MBRs to exist into two major groups according to their configurations; submerged and recirculated:

In the submerged arrangement, the membrane separation unit is submerged in the bioreactor tank. The driving force across the membrane is achieved a suction pump on the permeate line. Aeration and mixing are also achieved by the same unit. Biological degradation occurs in the mixed liquor around the membrane keeping all of the biomass within the reactor (Figure 9). Plate and frame or hollow fiber membranes are available of this configuration.

In the recirculated or external MBR, also called side-stream, the membrane separation unit is outside the main reactor. The biomass is separated externally, and returned to the reactor. A scheme of the recirculated MBR is presented in Figure 10. A high-flow recirculation pump is required for external configuration, the retentate is flowing through a tubular membrane or in-between flat sheet membranes in a cassette. Thus the power requirement is much higher for these membranes than in submerged systems

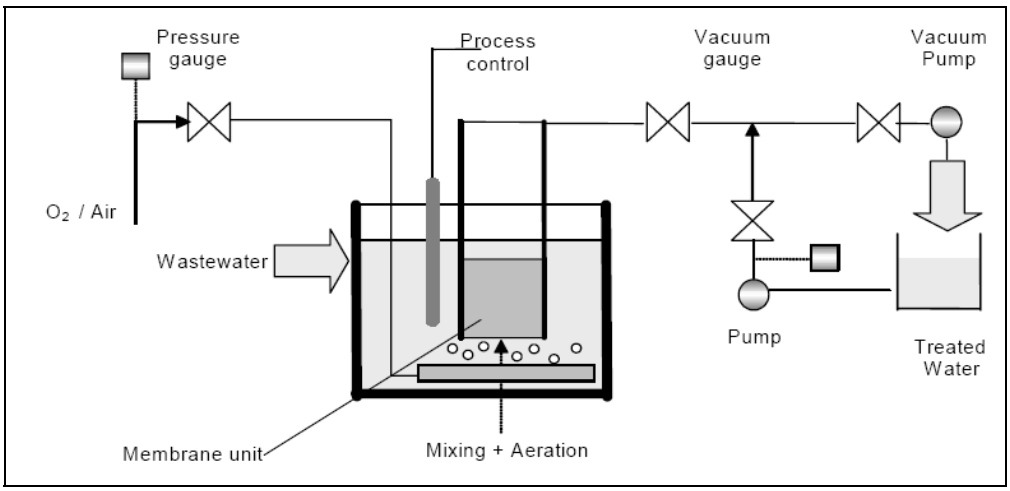

Figure 9 Submerged MBR Configuration - the membrane is situated inside the reaction vessel (Stephenson et al., 2000).

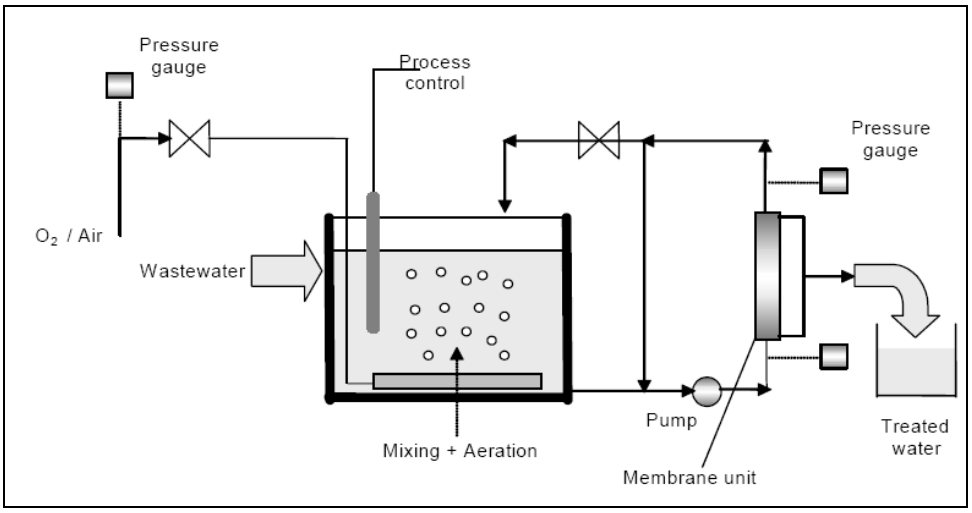

Figure 10 Recirculated or external MBR Configuration: the external MBR, the membrane is placed outside of the main reaction vessel (Stephenson et al., 2000).

2.6.3.3 Membrane materials and configurations

Membrane materials vary widely both in chemical composition and physical structure, but the most fundamentally important property is the mechanism by which separation is actually achieved. A more convenient practical categorization of membranes is according to the material composition, which is generally either organic (polymeric) of inorganic (mineral /ceramic and metallic). The physical structure of the membrane based on these materials can then vary according the exact nature of the material and/or the way in which it is processed. Examples of membrane materials are listed in Tables 7 and 8.

Table 7 Membrane materials by type (Stephenson et al., 2000).

Membrane Structure		Applications
Ceramic	0.1-10 μm pores	Microfiltration, gas separation, separation of isotopes
Etched polymers	0.5-10 μm cylindrical pores	Analytical and medical chemistry, sterile filtration
Supported liquid	Liquid-filled porous matrix	Gas separation, carrier-mediated transport
Symmetric microporous	0.05-5 μm pores	Sterile filtration, dialysis, membrane distillation
Integral asymmetric microporous	1-10 μm pores at membrane surface	Ultrafiltration, nanofiltration, gas separation, pervaporation
Composite asymmetric microporous	1-5 μm pores at membrane surface	Ultrafiltration, nanofiltration, gas separation, pervaporation
Ion exchange	Matrix of positive and negative charges	Electrodialysis

Table 8 Membrane materials by name (Stephenson et al., 2000).

Materials Advantage		Disadvantage
Titanium dioxide (TiO_2)	Good thermal resistance	Very expensive
Zirconium dioxide (ZrO_2)	Good Chemical resistance Good mechanical resistance	Limited to microfiltration and ultrafiltration Brittle materials
Cellulose acetate	Inexpensive Chlorine resistant Solvent cast	Poor thermal stability Poor chemical stability Poor mechanical stability
Polysulfone	Steam sterilizable pH resistant Solvent cast	Poor resistance to hydrocarbons
Polypropylene	Chemically resistant	Hydrophobic unless surface treated
Polytetrafluoroethylene (PTFE)	Very hydrophobic Excellent organic resistance Excellent chemical stability Sterilizable	Very hydrophobic Expensive
Polyamide	Good Chemical resistance Good thermal resistance	Sensitive to chlorine

Several types and configurations of membranes have been used for MBR applications. These include tubular, plate and frame, rotary disk, hollow fibers, organic (polyethylene, polyethersulfone, polysulfone, polyolefin, etc.), metallic, and inorganic (ceramic) microfiltration and ultra-filtration membranes. The pore size of membranes used ranged from 0.01 to 0.4 μm. The geometry of the membrane, i.e. in the way it is shaped, is crucial in determining the overall process performance. There are many principal configurations currently employed in membrane processes which all have various practical benefits and limitations (Table 9).

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Table 9 Advantages and disadvantages of current membrane configurations (Stephenson et al., 2000)

Configuration	Area/volume Ration (m^2/m^3)	Turbulence Promotion	Advantages Disadvantages		Applications (most important first)
Pleated cartridge	800-1,000	Very poor	robust construction compact design	Easily fouled cannot be cleaned	Dead end microfiltration
Plate-and-frame	400-600	Fair	can be dismantled for cleaning	Complicated design cannot be back-flushed	Electrodialysis, Ultrafiltration, Reverse osmosis
Spiral-wound	800-1,000	Poor	low energy cost robust and compact	Not easily cleaned cannot black-flush	Reverse osmosis Ultrafiltration
Tubular	20-30	Very Good	easily mechanically cleaned tolerates high TSS waters	High capital and membrane Replacement cost	Cross-flow filtration High TSS waters
Hollow fiber	5,000- 40,000	Very poor	can be back-flushed compact design tolerates high colloid levels	Sensitive to pressure shock	Ultrafiltration, Reverse osmosis

The hollow fibers are used in reverse osmosis to microfiltration, where the water flows from outside to inside the tubes, as well as from inside to outside especially in drinking water processes

The membranes primarily used in wastewater treatment are as follows

Plate and Frame – The plate and frame membranes consist of several flat sheets of membrane material, usually an organic polymer, stretched across a thin frame. The space between the membrane sheets is placed under vacuum in order to provide the driving force for filtration. Several plates are arranged in a cassette to allow to increase the filtration area in a convenient modular design. The membrane cassette is immersed in the mixed liquor and the separation flow is from outside-in.

Hollow fiber – Hollow fiber membranes consist of long bundles, or fibers, of hollow extruded membrane. They are most often of organic polymer. The fibers are potted in a supporting structure that serves as a manifold for permeate transport as well as an air delivery system. Similar to the plate and frame modules, air induced liquid crossflow prevents excessive cake formation and increases the lifespan of the membrane. The selectivity of the hollow fibres are often given by a very thin skin, smaller than 1 micron, which is deposited outside or inside the hollow fiber depending on the permeation mode out/in or in/out respectively. Some of them, called “double skin” membranes have a selective skin on the both sides.

Tubular –Tubular membranes are tubes. Below the membrane surface is a supporting structure with high porosity. In most cases, tubular membranes are made of inorganic material such as ceramic and have a metal oxide membrane surface to provide a small nominal pore size. A tubular membrane could be used in the inside-out arrangement with the feed water flowing along the center of the tube and the permeate passing to the outside walls, or the outside-in arrangement where the influent travels along the outside of the tube and travels axially inside.

2.6.3.4 Permeate flux and crossflow

The key elements of any membrane process are the influence of the following parameters on the overall permeate flux: the membrane resistance, the operational driving force per unit membrane area, the hydrodynamic conditions at the membrane/liquid interface, and the fouling of membrane surface. The flux is the amount of solvent and some components passing through a unit area of membrane per unit time. The flux is determined by both the driving force and the total resistance offered by the membrane and the interfacial region adjacent to it. The resistance of the membrane is fixed, unless it becomes partly clogged by components in the feed water.

In most membrane processes, there are three streams; a feed, a retentate and a permeate streams. The retentate stream is unpermeated products. If there is no retentate stream then operation is termed dead-end (Figure 11 top). Such operation is normally restricted to either low-solids water, such as cartridge filtration of boiler feed water. The alternative to dead-end operation is crossflow operation (Figure 11 bottom), in which the feed-water flows parallel to the membrane surface and so expediting the removal of accumulated material from the membrane. Crossflow operation then implies the existence of retentate stream. The more selective of membrane permeation, and larger the hydraulic resistance, the greater the propensity for crossflow rather than dead end operation.

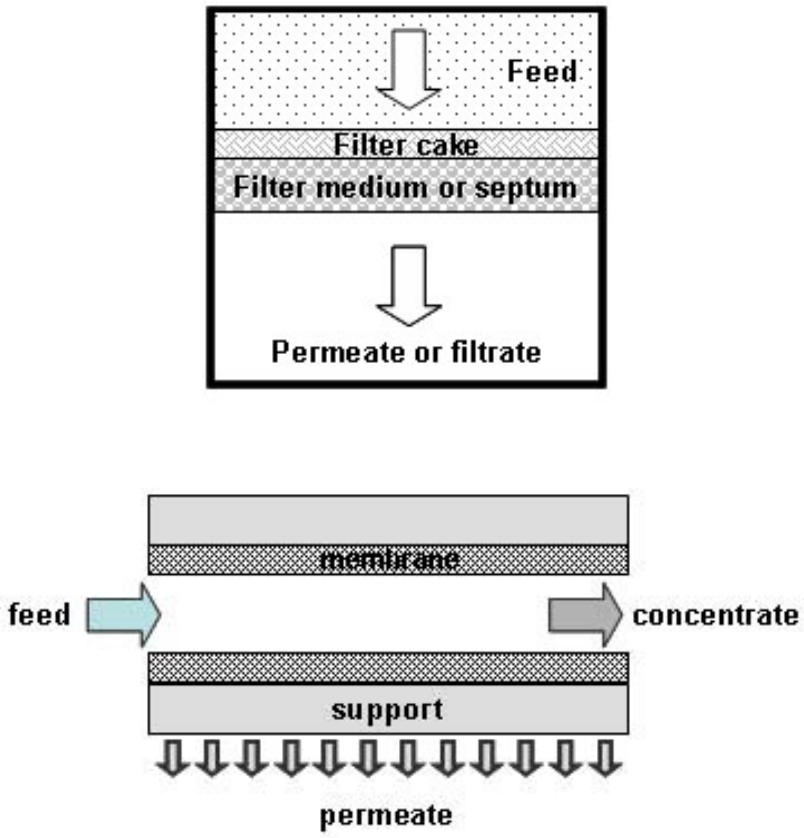


Figure 11 Dead-end (top) and cross flow microfiltration (bottom) (Stephenson et al., 2000).

2.6.3.5 Application of MBR for wastewater treatments

Recently, the membrane bioreactors (MBRs) has proved to be an attractive process for the treatment of industrial and municipal wastewaters as it prevents the loss of biomass from the digester and produces a more constant and better quality of final effluent (Fan and Huang, 2002; Fuchs et al., 2005; Pant and Adholeya, 2007; Satyawali and Balakrishnan, 2008). However, there are very few investigations on distillery wastewater treatment in an MBR. Some of these studies are descried in details as follow.

Choo and Lee (1996) examined a thermophilic (55^{O}C) membrane-coupled anaerobic bioreactor (MCAB) system using 20 kD polymeric plate and frame membrane modules in the external (recirculation) mode. This system was designed for the treatment of alcohol-distillery wastewater during a longtime operation. Enhanced COD removal was achieved with the complete retention of biomass either inside the anaerobic reactor or on the membrane surface. Membrane fouling was mainly attributed to external fouling, which was closely related to the movement of cells to the membrane surface and inorganic precipitation at the membrane surface

Membrane coupled anaerobic bioreactor using 0.2 μm polypropylene and 0.14 μm zirconia skinned inorganic tubular membranes has also been investigated for the treatment of 40,000 mg/l COD distillery wastewater at 55^{O}C. High COD removal (90%) was observed in both the anaerobic MBRs (Kang et al., 2002). In addition, inorganic membrane was found to have accumulated inside the membrane pore and played a key role in flux decline. For the organics, however, a thick cake layer composed of biomass formed on the membrane surface, thus causing a major hydraulic resistance.

Zhang et al. (2006) reported aerobic treatment of simulated distillery wastewater (10,000 mg/l COD) at 30-45^{O}C using 0.2 μm stainless steel membranes. With a HRT of 10–30 h and a VLR of 0.6–2.8 kgCOD·m^{-3}·h^{-1}, mean COD and TN removal efficiencies were 94.7% and 84.4%, respectively.

The reported COD removal efficiencies with mesh/cloth-based MBRs are usually high, but the studies are limited to low influent COD (<2000 mg/l). Fan and Huang (2002) reported 84% COD removal efficiency for a mesh filter MBR operating on municipal wastewater (97.9–371.7 mg/l COD). Similarly COD removal (92%) was observed with a nylon mesh-based MBR operating on municipal wastewater having a COD of 270–570 mg/l (Fuchs et al., 2005). Over 95% COD removal efficiency was observed for food processing wastewater (800 –1,800 mg/l COD) in an MBR

equipped with non-woven fabric having 20 μm pore size (Chang et al., 2007). Furthermore, the BOD:COD ratio of this effluent is relatively low (Satyawali, M. Balakrishnan (2008) due to the presence of recalcitrant organics and growth inhibiting substances (Pant and Adholeya, 2007).

Satyawali and Balakrishnan (2008) investigated operation of a laboratory scale membrane bioreactor (MBR) in the continuous mode for distillery wastewater treatment using submerged 30 μm nylon mesh filters. The study involved acclimatization of municipal activated sludge in a fed-batch reactor followed by operation in a continuous mode at organic loading rates ranging from 3 to 5.71 kg /m^3/day. Up to 41% COD removal was obtained over 245 days of reactor operation, however high molecular weight compounds comprising the color imparting melanoidins remained unaffected. Up to 100% suspended solid retention was obtained and the system could be operated up to 2 weeks without significant flux drop.

CHAPTER III

MATERIALS AND METHODS

3.1 Molasses wastewater

Molasses wastewater was obtained from Sangsom Co., Ltd., a alcoholic distillery plant from sugarcane molasses in Nakornpathom Province, Thailand. In the laboratory, the effluent samples were stored at $4^{O}C$. The effluent was characterized and analyzed for pH, chemical oxygen demand (COD), biological oxygen demand (BOD) based on the Standard Methods for Examination of Water and Wastewater (APHA, 1998). The characteristics of molasses wastewater were indicated in Table 3.1.

Table 3.1 Characteristics of molasses-based distillery wastewaters from Sangsom Co., Ltd

Water Quality index	Slop wastewater
Temperature	95-100^{O}C
pH value	3.8-4.5
TDS	7,600 mg/l
Suspended Solid	15,000 mg/l
Color and odor	Dark brown / bad smell
BOD	35,000 mg/l
COD	100,000 mg/l

3.2 Preparation of molasses wastewater medium

After experiments presented in the chapter 4, three different media were kept for studying the decolorization of molasses wastewater by a bacterial consortium. They were prepared as follow:

- *Molasses wastewater medium (WW)* was made by diluting molasses wastewater from alcoholic distillery with distilled water to 20% (v/v).
- *Molasses wastewater-containing LB medium (LBWW)* was LB medium containing 20% (v/v) of molasses wastewater from alcoholic distillery
- *Modified molasses wastewater medium (MM)* consisted of (20%, v/v) of molasses wastewater from alcoholic distillery, 0.01% (w/v) $NaNO_3$, 0.2% (w/v)

K_2HPO_4, 0.1% (w/v) KH_2PO_4, 0.01% (w/v) $MgSO_4 \cdot 12H_2O$, 2% (w/v) glucose and 0.1% (w/v) yeast extract.

3.3 Preparation of different melanoidins-containing wastewater media

Three different media with different sources of melanoidins were prepared. Melanoidins-containing solutions were used including sugarcane molasses wastewater, beet molasses wastewater and Viandox sauce. Other components of the each medium were as follows: 0.01% (w/v) $NaNO_3$, 0.2% (w/v) K_2HPO_4, 0.1% (w/v) KH_2PO_4, 0.01% (w/v) $MgSO_4 \cdot 12H_2O$, 2% (w/v) glucose and 0.1% (w/v) yeast extract, and the initial pH was adjusted to 4.

The characteristics of each medium were indicated in Table 3.2. The sugarcane molasses wastewater was obtained from SangSom distillery, Nakhon-Pathom province, Thailand. Beet molasses wastewater and Viandox sauce were obtained from Laboratoire de Génie Chimique, Toulouse, France.

Table 3.2 Characteristics of synthetic melanoidins-containing wastewater

Color substances	Initial concentration (%, v/v)	OD_{475}	COD (g/L)
Viandox	13.5	5.71	22.8
Beet molasses wastewater	41.5	5.90	30.75
Sugarcane molasses wastewater	20	5.71	21.6

3.4 Screening of molasses-decolorizing bacterial consortium

Various bacterial consortia were isolated from natural environments and wastewater treatment plant.

Several samples including soils, sediments and wastewaters were collected from various sources in Thailand to isolate a bacterial consortium which has high molasses-decolorization power. In order to enrich molasses-decolorizing population, five milliliters of each sample were transferred into 50 ml of LB medium in Erlenmeyer flasks and cultivated at 30^{O}C on a rotary shaker at 200 rpm.

For the first step of screening, the inoculum of enriched bacterial consortia, prepared by growing in LB broth under shaking at 200 rpm at 30^{O}C for 24 hours, were loaded into holes drilled with a cork borer (0.7 cm in diameter) on modified molasses wastewater (MM) agar plate. Sterile LB medium was used as control. Decolorization was observed when a clear zone appeared around the holes after incubation at room temperature for 48 h under either aerobic. Bacterial consortia which showed high molasses decolorization in the primary screening were cultured in Erlenmeyer flask containing modified molasses wastewater medium (MM) at 30^{O}C on rotary shaker at 200 rpm.

The bacterial consortium was chosen for its ability to decolorize the medium. It exhibited the highest melanoidin decolorization efficiency (20% color reduction) within 48 h under aerobic conditions and this consortium was selected for further study. The bacterial consortium samples were stored at 0^{O}C in LB-broth supplemented with (15%, v/v) of glycerol until used in the experiments.

3.5 Construction of bacterial consortia for optimal decolorization

In order to find a bacterial consortium efficient for the color removal in mixed cultures, experiments were performed with various combinations of bacteria, namely *Klebsiella oxytoc*a (T1), *Serratia mercescens* (T2), *Citrobacter* sp. (T3) and unknown bacterium DQ817737 (T4).

For construction of the active bacterial consortia, a loopful of each bacterium (T1, T2, T3 and T4) from LB plate was precultured in 50 ml LB at 30^{O}C under shaking at 200 rpm. After 24 h, bacterial cells of each strain were harvested by centrifugation at 10,000 rpm at 4^{O}C for 10 min then washed with sterile normal saline solution. Washed bacterial cells at appropriate volume were subsequenctly inoculated into fresh synthetic melanoidins-containing wastewater media to obtain an initial OD_{600} of 0.2. Several consortia comprising of different bacterial compositions were constructed at the same initial cell density.

3.6 Inocula preparation

3.6.1 Decolorization of different melanoidins-c ontaining w astewater media

For decolorization of the different melanoidins-containing wastewaters, the inoculum was prepared by transferring the bacterial consortium into a flask containing 50 ml LB medium and incubated for 24 h under shaking (200 rpm) at

30^{O}C. 10% inoculum was transferred into shake flasks containing 250 ml of melanoidins-containing wastewater medium, each flask containing one different colored substance. The consortium was then incubated under shaking conditions (200 rpm) at pH 4, 30^{O}C.

3.6.2 Study of the optimal decolorization

The bacterial consortium was transferred into 250 ml Erlenmeyer flasks containing 50 ml synthetic melanoidins-containing wastewater medium, using 2% (v/v) Viandox as color substance. Incubation was carried out under shaking at 200 rpm, 30^{O}C for 48 h. Bacterial cells were harvested with 3 h intervals by centrifugation at 10,000 rpm, 4^{O}C for 10 min.

3.6.3 Study on limitation of decolorization

The bacterial consortium was inoculated into melanoidins-containing wastewater medium and cultivated under shaking (200 rpm) at 30^{O}C for 48 h. Cells were harvested by centrifugation (10,000 rpm, 10 min, 4^{O}C) and washed three times successively with sterile normal saline solution in order to eliminate the residual culture medium. Washed bacterial cells were resuspended in the fresh culture medium of the same volume and cultivated under condition as described above.

Meanwhile, the used culture medium was centrifuged again at 10,000 rpm for 10 min at 4^{O}C to completely remove the bacterial cells, then inoculated with fresh bacterial cells (10% w/v) and cultivated under the same condition as described above.

3.7 Identification of effluent decolorizing consortium

3.7.1 Bacterial identification by 16s rDNA sequence

On the basis of morphological study, the bacterial strains present in enriched bacterial consortium were isolated into pure culture. A loopful of each strain taken from an isolated colony on LB plate was precultured in 50-ml Bacto LB broth (in g l^{-1}; 10 tryptone, 5 yeast extract, and 10 NaCl) for 12 hours. The culture broths were centrifuged at 10,000 rpm for 2-5 min and the cell pellets were resuspended in 100-500 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Bacteria were lysed by boiling for 10-15 min.

DNA solutions were separated by centrifugation at 10,000-12,000 rpm for 5-10 min and used as DNA template for 16S rDNA amplification. Extracted DNA was

amplified by using PCR and the universal 16S rDNA primers 20F (5'-GAG TTT GAT CCT GGC TCA G-3') and 802R (5'-TAC CAG GGT ATC TAA TCC-3'). The PCR reaction was run with PCR Buffer pH 8.8 (Biolab containing: 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_2$ and 0.1% Triton X-100), 0.4 µM of deoxynucleotide triphosphate (dNTP), 0.4 µM of each primer and 1 U of *Taq* polymerase (Biolab). The initial cycle consisted of 5 min at 95^{O}C and was followed by 25-30 cycles of 95^{O}C for 1 min, 60^{O}C for 1 min, and 72^{O}C for 1 min. The final cycle for extension step consisted of 72^{O}C for 3 min. The PCR products from 20F and 802R primers were used as template for PCR based DNA sequencing. For increasing the specificity of DNA product, UFUL primer (5'-GCCTAACACATGCAAGTCGA-3') was used in DNA sequencing reaction (Nilsson et al., 2003). The DNA sequencing reaction solution contained Bigdye Termination v3.1 cycle sequencing kit (Master Mix) and 3.2 pM of UFUL primer. The initial cycle consisted of 5 min at 95^{O}C and followed by 30 cycles of 95^{O}C for 30 s, 50^{O}C for 10 s, and 60^{O}C for 4 min. The final cycle for extension step consisted of 60^{O}C for 4 min. 62.5 µl of absolute ethanol, 3.0 µl of 3M sodium acetate pH 4.6 and 14.5 µl of distilled water were added to resulting amplicons and they were incubated at room temperature for 15 min. Then amplicons were centrifuged at 12,000 rpm, room temperature for 15 min and washed with 70% ethanol. The amplicons were dried at 95^{O}C for 2-3 min and resuspened with 10-15 µl of Hi-Di Formamide. Samples were boiled at 95^{O}C for 2 min and cooled down for 5 min. Samples were sequenced by Automated DNA Sequencer. The sequences of 16S rDNA were compared with those available in the GenBank, EMBL, and DJB databases using the gapped BLASTN 2.0.5 program through the National Center for Biotechnology Information.

3.7.2 Analysis of microbial community using denaturing gradient gel electrophoresis (DGGE) of 16S rDNA.

DNA was directly extracted from bacterial culture with bead-beating instrument and UltraCleanTM Soil DNA Kit (MO BIO Laboratories, Inc).

Then, each DNA sample was amplified by 16S rDNA PCR technique using PRBA 338F+CG clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG-3') and PRUN518R (5'-ATT ACC GCG GCT GCT GG-3') primers. The initial cycle consisted of 5 min at 94^{O}C and followed by 20 cycles of 94^{O}C for 1 min, 65^{O}C for 1 min, and 72^{O}C for 2 min. After that, each sample went trough 30 cycles of 94^{O}C for 1 min, 55^{O}C for 1 min, and 72^{O}C for 2 min. The final cycle for extension step was at 72^{O}C for 10 min.

PCR product was subsequently run on 8% polyacrylamide gel with a denaturing gradient of urea and formamide denaturant ranging from 25-60% for 5 hr

at 130 volts with 1xTAE. DGGE gel was stained in 50 µg per ml of ethidium bromide for 20 minutes.

DNA band profiles can be detected under the UV transilluminater (Cindy et al., 2000; Edenborn and Sexstone, 2007).

3.8 Device for the decolorization in membrane bioreactor

The molasses wastewater treatment by bacterial consortium was run continuously in membrane bioreactor. The schematic diagram of membrane bioreactor was illustrated in Figure 3.1. A cross flow membrane filtration set with a total area of 0.013 m^2 was installed in the treatment system. The membrane bioreactor process was carried out in 2L of reactor with working volume of 1.6L at 30^{O}C with sludge retention time (SRT) of 50 days and hydraulic retention time (HRT) at 20 and 40 h, respectively. The aeration was maintained at 0.1 vvm with agitation speed at 150 rpm.

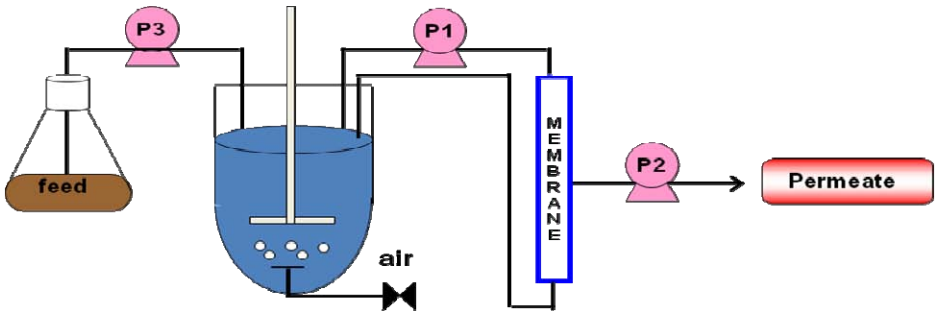


Figure 3.1 Schematic diagram of wastewater treatment using MBR.

3.9 Analytical methods

3.9.1 Measurement of bacterial growth

Bacterial growth was determined by direct and indirect methods depending on available equipment in Thai or French laboratory.

1. **Viable plate count method**

Sample of cell suspension was diluted and spread (volume to be specified) over the surface of a growth medium, then, the plates were incubated at 30^{O}C. The

colonies were counted after 24h. Total number of colonies is determined. Each plate should have 30-300 colonies at least.

2. Spectrophotometry

Optical density (OD) was also used to monitor the bacterial growth in this study. The absorbance was measured at 600 nm by using Hitachi spectrophotometer, model U-2000. The bacterial density (C*) was calculated using Equation; $C^* = C_1 - C_O$; where C_1 is the OD value of the culture broth; and C_O is the OD value of supernatant obtained after centrifugation of the culture broth.

3. Dry weight technique.

Cells in suspension were collected by centrifugation (10,000 rpm for 10 min at 4^OC), washed with distilled water, and dried in an oven at 80^OC until getting a constant dry weight.

3.9.2 Melanoidins color determination

The color intensity of melanoidins-containing wastewater was determined by measuring optical density (OD) at 475 nm of the supernatant obtained upon centrifugation (10,000 rpm for 10 min at 4^OC) of 5 ml culture by using Hitachi spectrophotometer, model U-2000.

3.9.3 Chemical oxygen demand (COD) analysis

The COD content was determined by a spectrophotometric method using Hach COD reagent test kit (HACH Company, USA). See appendix 1 for details.

3.9.4 Total nitrogen analysis

The total nitrogen was determined by a spectrophotometric method using Hach Total nitrogen reagent test kit (HACH Company, USA). See appendix 1 for details

CHAPTER IV

RESULTS

4.1 Screening of molasses wastewater-decolorizing bacterial isolates

Samples collected from various sources in Thailand, e.g. soils, sediments, and alcoholic distillery effluents, were screened for bacterial isolates capable of decolorizing sugarcane molasses wastewater. Potential bacterial isolates were selected on the basis of rapid molasses decolorization. All isolates were tested for molasses decolorization using the agar plate method, as primary screening, and the shaking culture method, as secondary screening, described as follow.

4.1.1 Primary screening

Five ml of water sample or 5 g of soil samples were added to 50 ml LB broth and incubated at 30^{O}C on rotary shaker 200 rpm for 48 h. Enriched samples were then plated onto MM agar plates and incubated for 48 h at 30^{O}C. All samples were observed for molasses decolorization: a clear zone appeared around the bacterial colony.

After 48 h of incubation, it was found that 90 bacterial isolates showed the clear zone around their colonies on the agar plate (Fig. 4.1). It meant that these isolates showed molasses decolorization and the details of these isolates are shown in Table 4.1. All isolated colonies showing molasses decolorization on MM agar plate were purified by streaking technique and maintained on MM agar slants at 4^{O}C.

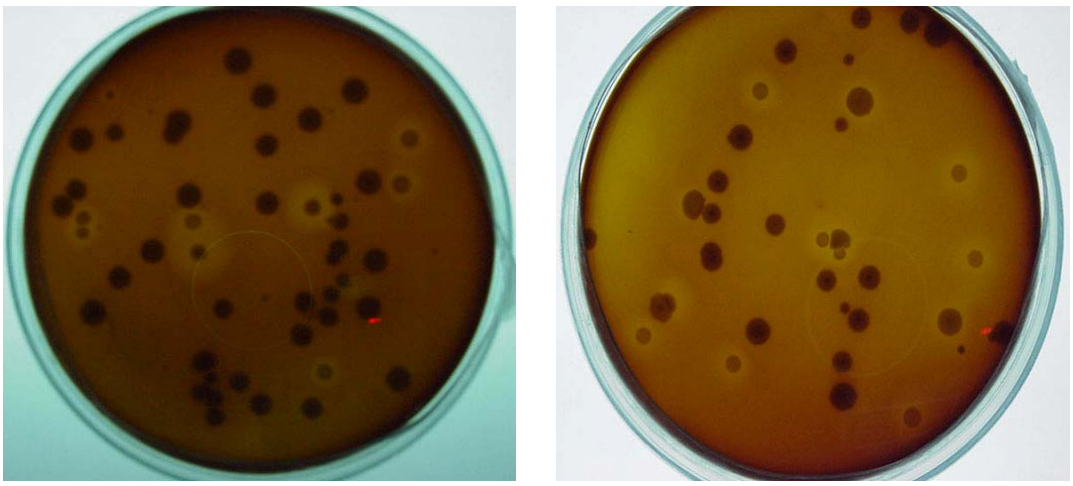


Figure 4.1 Characterization of bacterial colonies on MM agar plates under aerobic incubation for 48 h.

Table 4.1 Primary screening for molasses-decolorizing bacteria.

	Samples	**Number of decolorizing bacteria**	**Isolates**
1	Soil sample 1 from Sangsom Co., Ltd.	4	A1, A2, A5 and A6
2	Soil sample 2 from Sangsom Co., Ltd.	-	-
3	Soil sample 3 from Sangsom Co., Ltd.	3	C2, C3 and C4
4	Slop sample 1 from Sangsom Co., Ltd.	5	D1-D5
5	Slop sample 2 from Sangsom Co., Ltd.	37	E1-E4, E5 and E6-E37
6	Soil sample from Nakonrachasrima Province	4	F1, F2, F3 and F8
7	Soil sample from Srinan national park, Nan Province	4	G1-G4
8	Soil sample 1 from Nan Province	7	H1-H7
9	Soil sample 2 from Nan Province	-	-
10	Soil sample 3 from Nan Province	5	J1-J5
11	Soil sample 4 from Nan Province	4	K1-K4
12	Soil sample 5 from Nan Province	4	L1-L4
13	Soil sample 6 from Nan Province	3	M1-M3
14	Soil sample 7 from Nan Province	-	-
15	Soil sample 8 from Nan Province	-	-
16	Soil sample 9 from Nan Province	6	P1-P6
17	Soil sample from Pukraduang, Leay Province	4	Q1-Q4
Total (isolates)		90	

4.1.2 Secondary screening

In order to select the effective bacterial isolates, the second step of screening was performed. Ninety isolates which showed molasses decolorization in the first step were cultured using the liquid culture method with MM medium. Each isolate was cultured in 50 ml of medium in 250 ml Erlenmeyer flask on rotary shaker at 200 rpm, $30^{O}C$ for 2 days. Subsequently, the culture broths were collected and centrifuged at 10,000 rpm, $4^{O}C$ for 10 min. The supernatants were determined for the decolorization by measurement of optical density (OD) of the supernatant at 475 nm. Decolorization of molasses wastewater in MM medium by 90 isolates is shown in Table 4.2.

From the secondary screening, it was found that 26 out of 90 isolates showed molasses decolorization. Only ten isolates i.e. E5, E15, E22, F2, F3, F8, G3, G4, P3 and P4, showed decolorization higher than 10% when compared with control. The isolate E5, which was isolated from slop sample from Sangsom Co.Ltd. alcoholic distillery, showed the highest molasses decolorization at 18.8%. It was selected for further investigations.

Table 4.2 Secondary screening for molasses-decolorizing bacteria

Sample	Decolorization (%)	Sample	Decolorization (%)	Sample	Decolorization (%)
A1	0.00	E19	0.00	H4	0.00
A2	0.52	E20	0.00	H5	0.00
A5	0.00	E21	0.00	H6	0.89
A6	0.00	E22	14.24	H7	0.89
C2	0.00	E23	0.00	J1	0.00
C3	0.00	E24	0.00	J2	0.00
C4	0.00	E25	0.00	J3	0.00
D1	0.00	E26	0.00	J4	0.00
D2	0.00	E27	0.00	J5	0.00
D3	0.00	E28	0.00	K1	0.00
D4	0.00	E29	0.00	K2	1.44
D5	0.00	E30	0.00	K3	1.80
E1	0.00	E31	0.00	K4	3.72
E2	2.42	E32	0.00	L1	0.00
E3	3.87	E33	0.00	L2	0.00
E4	1.54	E34	0.00	L3	0.00
E5	**18.80**	E35	0.00	L4	0.00
E6	0.00	E36	0.00	M1	0.00
E7	2.59	E37	0.00	M2	0.00
E8	2.23	F1	0.00	M3	0.00
E9	4.53	F2	16.75	P1	0.00
E10	0.64	F3	13.38	P2	0.00
E11	0.00	F8	10.30	P3	13.29
E12	1.17	G1	0.00	P4	13.45
E13	1.59	G2	0.00	P5	0.00
E14	2.08	G3	11.85	P6	0.00
E15	**13.64**	G4	12.54	Q1	0.00
E16	0.00	H1	0.00	Q2	0.00
E17	0.00	H2	0.00	Q3	0.00
E18	0.00	H3	0.00	Q4	0.00

4.1.3 Investigation of decolorization stability of bacterial isolates E5

Decolorization stability of bacterial isolate E5 was then confirmed through an experiment run in MM broth under shaking conditions. The colony of isolate E5 grown on MM agar plate (Fig. 4.2) was inoculated into MM broth and incubated under aerobic conditions for 48 h. Subsequently, 5 ml aliquots from the bacterial culture were transferred to fresh MM broth and incubated under aerobic conditions for 48 h. After 5 consecutive subcultures, the decreasing decolorization trend of molasses wastewater was observed (Fig. 4.3). The decolorization of molasses wastewater in MM broth by the isolates E5 dropped to 2.3%. In addition, there was observed a slight manifestation of varying decolorization zones around colonies of isolate E5 on MM agar.

Figure 4.2 Bacterial isolate E5 colonies, and clear zones on MM agar

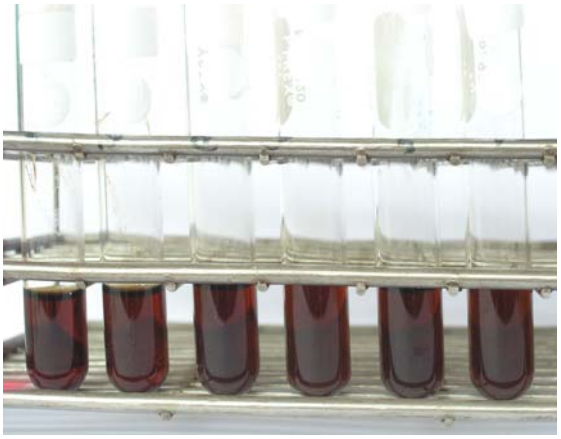


Figure 4.3 MM broth after 5 consecutive subcultures of the bacterial isolates E5.

It demonstrated that pure culture of bacterial isolate E5 displayed limited ability to decolorize molasses wastewater in long-term. Improvement of its culture conditions for further decolorization processes was required. Similarly, it was previously established that bacteria, especially pure culture, displayed limited ability to decolorize melanoidins because of the toxicity of metabolites which were formed and accumulated during molasses pigment decolorization. These metabolites thereby repressed the efficiency of bacterial cells (Raghukumar et al., 2004).

Therefore, the application of bacterial consortium might be a more promising strategy for decolorization molasses wastewater by biological treatment. Since bacterial members of consortium might have different metabolic activities, a bacterial consortium could be highly effective in decolorizing a broad spectrum of structurally diverse melanoidins (Manjinder et al., 2005; Sarayu et al., 2008).

4.2 Screening of molasses wastewater-decolorizing bacterial consortium

The purpose of using a consortium of bacteria as inoculum for molasses decolorization was to increase decolorization and to minimize instability problem of single bacterium by providing synergistic decolorization mechanisms. Therefore, screening of different bacterial consortia was carried out to select the most suitable sample for optimization studies.

Various bacterial consortia from 21 sources were enriched in 50 ml of LB broth and cultivated at 30^{O}C on a rotary shaker at 200 rpm. As described in chapter 3, a primary screening was carried out. Enriched bacterial consortia were loaded into holes on MM agar plates. The sterile LB medium was used as control. When decolorization was observed, a clear zone appeared around the holes after incubation at 30^{O}C for 48 h under aerobic conditions.

The results of primary screening showed that 9 different bacterial consortia were capable of decolorizing molasses wastewater under aerobic conditions. Clear zones were observed around the holes on MM agar plate (Fig. 4.4). Subsequently, the ability to decolorize molasses of those 9 consortia was re-tested in MM medium with secondary screening. The consortia were cultured in the MM broth at 30^{O}C on rotary shaker at 200 rpm for 48 h. The molasses decolorization was determined by measurement of optical density of the supernatant at 475 nm.

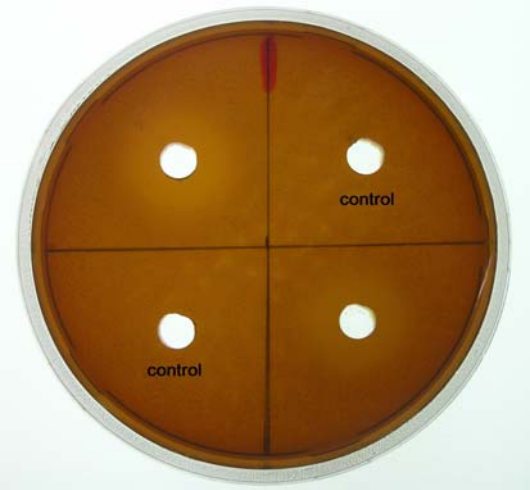


Figure 4.4 Characterization of holes on MM agar plate after incubation of bacterial consortia under aerobic conditions for 48 h.

The overall decolorization profile of the different consortia is shown in the Table 4.3. In comparison with other consortia, maximum decolorization was obtained from CONS8, which was the bacterial consortium enriched from waterfall sediments in Maehongsorn province. This consortium was selected for further study due to its highest decolorization of 20.02% when cultivated in the MM medium under aerobic conditions for 48 h.

Table 4.3 Primary and secondary screening for molasses-decolorizing bacterial consortia

	Samples	Bacterial consortium	Decolorization (%)
1	Soil sample 1 from Sangsom Co.Ltd.	CONS1	0.00
2	Soil sample 2 from Sangsom Co.Ltd.	-	-
3	Soil sample 3 from Sangsom Co.Ltd.	CONS2	0.00
4	Slop sample 1 from Sangsom Co.Ltd.	-	-
5	Slop sample 2 from Sangsom Co.Ltd.	CONS3	17.80
6	Soil sample from PVD, Nakonrachasrima Province	CONS4	11.97
7	Soil sample from Srinan national park, Nan Province	CONS5	5.64
8	Soil sample 1 from Nan Province	-	-
9	Soil sample 2 from Nan Province	-	-
10	Soil sample 3 from Nan Province	-	-
11	Soil sample 4 from Nan Province	CONS6	3.44
12	Soil sample 5 from Nan Province	-	-
13	Soil sample 6 from Nan Province	-	-
14	Soil sample 7 from Nan Province	-	-
15	Soil sample 8 from Nan Province	-	
16	Soil sample 9 from Nan Province	CONS7	13.79
17	Soil sample from Pukraduang, Leay Province	-	-
18	Water sample from Mohpang waterfall, Maehongsorn province	-	-
19	Waterfall sediment sample from Mohpang waterfall, Maehongsorn province	CONS8	20.02
20	Black sand, Chanthaburi Province	CONS9	15.43
21	Sea water of black sand beach, Chanthaburi Province	-	-
Total 9			

4.3 Optimization of culture conditions for decolorization

Decolorization of the bacterial consortium CONS8 was investigated in the different media and under different culture conditions.

The consortium was cultivated in LB medium under aerobic conditions at $30^{O}C$. After 24 h incubation, the medium was then centrifuged at 10,000 rpm, $4^{O}C$ for 10 min, washed twice and re-suspended in 0.85% NaCl to obtain an OD_{600} of 1. The decolorization experiments were carried out by transferring washed bacterial cells into test tubes containing individual culture medium as follow; molasses wastewater (WW), LB medium with molasses wastewater (LBWW) and modified molasses wastewater (MM), respectively. To examine the effect of initial pH and aeration on the decolorization, culture media (WW, LBWW and MM) were prepared at pH 4, 7 and 9 and the bacterial cells were inoculated and cultivated under different aeration conditions aerobic (agitation at 200 rpm), facultative (without agitation) and anaerobic (CO_2). The schematic diagram of this experiment is shown in Figure 4.5.

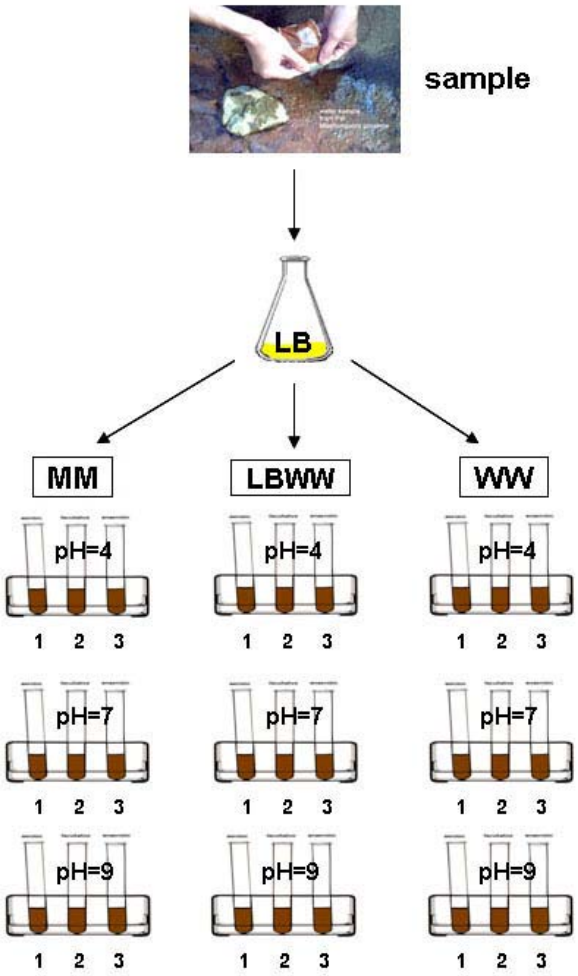


Figure 4.5 Schematic diagram of optimization experiment

Figure 4.6 (A-C) showed the growth of the bacterial consortium after incubation in 3 culture media at pH4 under aerobic, facultative and anaerobic condition. It was shown that bacterial growth was very low in all culture media and culture conditions, especially at pH4 (Fig. 4.6A). The growth of bacterial consortium in MM medium at pH7 under anaerobic condition was higher than any other media and cultured condition (Fig. 4.6B). The maximum bacterial growth in LBWW medium was found under aerobic condition at pH9 (Fig. 4.6C). However, the growth of bacterial consortia in WW medium was very low in all culture conditions.

Figure 4.7 (A-C) showed the molasses-decolorizing activity of the bacterial consortium on different media and different pH. The results showed that the decolorizing activity of bacterial consortium incubated in LBWW medium at pH4 under anaerobic conditions had the highest decolorizing activity approximately 26.5% (Fig. 4.7A). In contrast, only 4.8% was observed using under the same culture conditions in WW medium. Some nutrients and salts necessary for promoting growth and decolorization by the consortium might be lacking in WW medium. It was also found that the decolorization by the consortium incubated in MM medium was higher than other culture media under facultative and aerobic conditions. Decolorization efficiency in MM medium was enhanced up to 25.5% in 48 h under facultative conditions at pH4 whereas cell concentration was very low.

At pH7, the maximum decolorization of 23.8% was also found in MM medium under anaerobic conditions (Fig. 4.7B), whereas the consortium showed only 5% decolorization of MM medium under aerobic conditions at the same pH. At pH9, the highest decolorization of 20.4% was achieved in MM medium under facultative condition (Fig. 4.7C).

The higher color removal at acidic pH observed in this study might be due to the fact that melanoidins responsible for color were less soluble in acidic pH than in alkaline pH (Miranda et al., 1996). Hence, in the acidic pH, the melanoidins might be precipitated and removed more easily.

The difference in decolorization among various culture media might be linked to the fact that molasses wastewater was deficient in carbon content so biodegradation without any extra carbon source was found to be very difficult. Its recalcitrance was also due to presence of melanoidins, brown colored substances, which are formed by Maillard amino carbonyl reaction. Hence, supplementation with labile carbon sources appeared to be necessary for decolorization of molasses wastewater by bacterial consortium. Kambe and his co-workers reported a maximum color removal of molasses (A_{475} = 7.0) of 35.5% by *Bacillus smithii* at 55^{O}C under anaerobic conditions in presence of either peptone or yeast extract as supplemental nutrient while this strain could not use molasses wastewater as sole carbon source (Kambe et al., 1999). Kumar and Chandra have also reported that the addition of 1% glucose as a supplementary carbon source was necessary for molasses decolorization of a modified GPYM medium containing melanoidins (10% volume by volume) by *Bacillus thuringiensis*, *Bacillus brevis*, and *Bacillus* sp. up to 22%, 27.4%, and 27.4%, respectively (Kumar and Chandra, 2006). Acetogenic bacteria strain No.BP103 could also decolorize 73.5% of molasses pigments (A_{475} = 3.5) in molasses wastewater medium supplemented with glucose, yeast extract, and basal

mineral salts whereas the decolorization with this strain was dramatically decreased to only 9.75% in the absence of nutrient supplement (Sirianuntapiboon et al., 2004a). Similar result was also observed on the decolorization by bacterial consortium DMC which achieved a maximum molasses decolorization of 67% using basal medium containing distillery spent wash (A_{475} = 2.8-3) in the presence of 0.5% glucose (Mohana et al., 2007).

In general, several microorganisms that have been shown to degrade melanoidins under anaerobic condition are not suitable for treating effluent from molasses based distilleries. It is possible that they are lack of oxygen, which is necessary for oxidative degradation of melanoidins, in the effluent. However, the results presented in this study showed that color removal under facultative and anaerobic conditions were higher than under aerobic conditions. Hence, the decolorization mechanisms of molasses wastewater by bacterial consortium in this study might result from 2 possible mechanisms. One might be due to the color adsorption by bacterial cell and the other to the metabolism of bacteria under facultative and anaerobic conditions such as fermentation and anaerobic respiration.

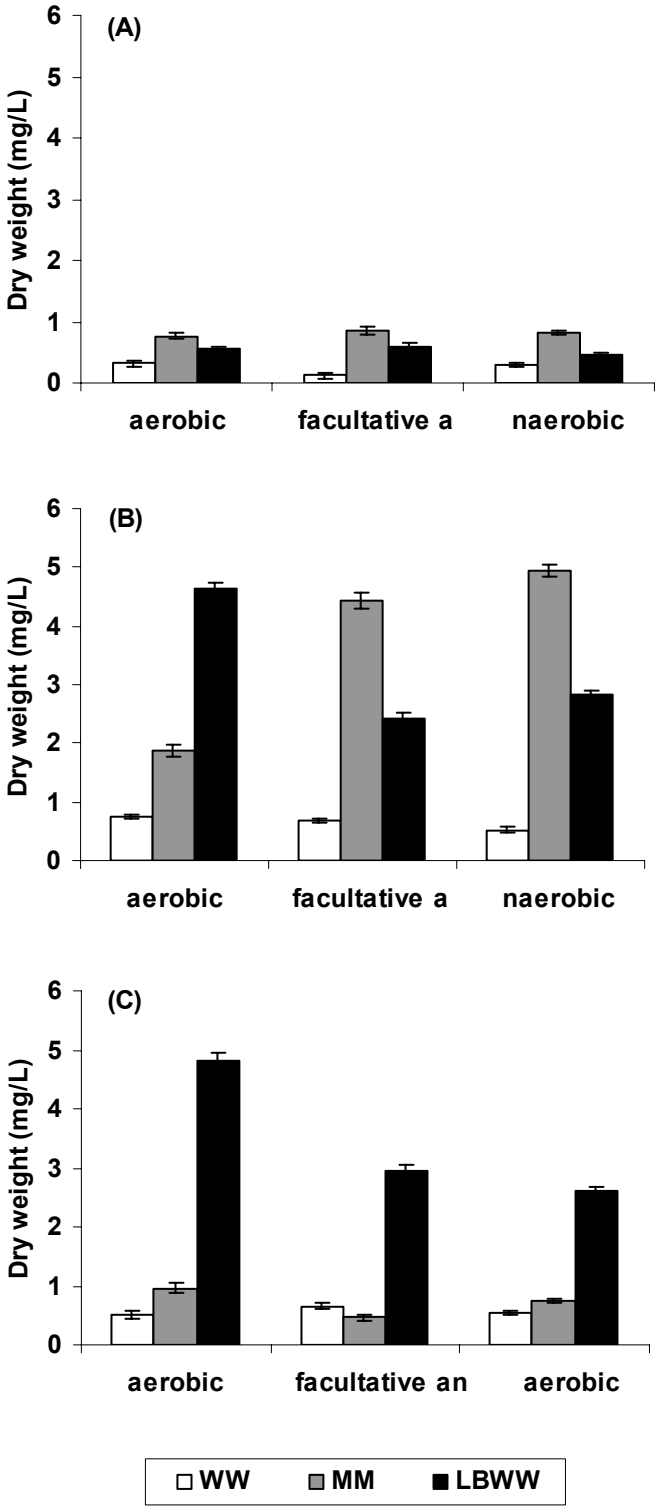


Figure 4.6 Effect of medium composition and culture conditions on growth of bacterial consortium CONS8 at pH4 (A), pH7 (B), and pH9 (C).

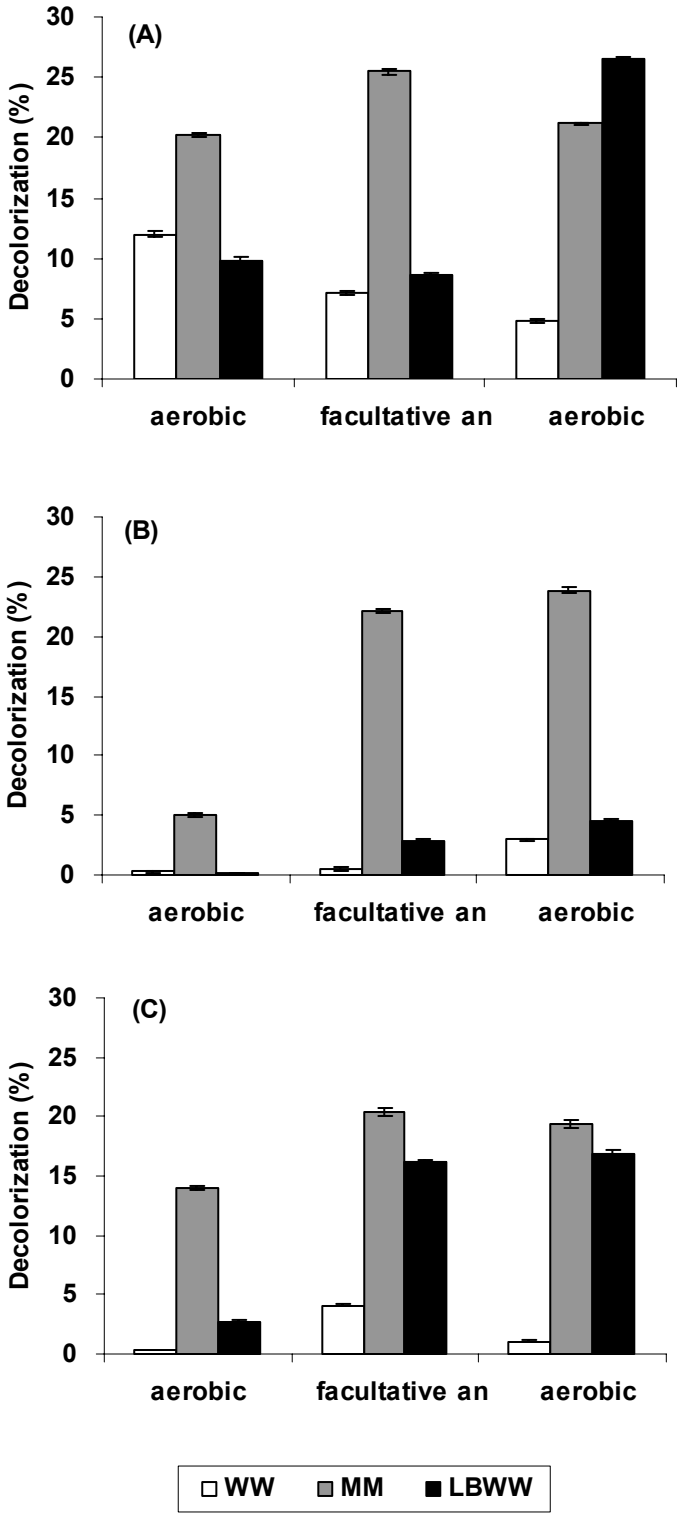


Figure 4.7 Effect of medium compositions and culture conditions on decolorization of molasses wastewater by bacterial consortium CONS8 at pH4 (A), pH7 (B), and pH9 (C).

4.4 Time course of growth and decolorization of the bacterial consortium CONS8

In the study dealing with optimization of culture conditions, our results demonstrated that the bacterial consortium gave the highest decolorizing activity in LBWW medium, pH4 under anaerobic conditions. However, LBWW medium might be never accepted in term of application due to excessive chemical use and high cost. Thus, MM medium was selected for use in the following study. Among MM medium, the bacterial consortium showed the highest decolorization at pH4 under facultative conditions. Unfortunately, facultative conditions are not suitable for application in conventional molasses wastewater treatment, which is mainly aerobic or anaerobic classical process.

Aerobic treatment systems have been demonstrated efficient for the treatment of various kinds of wastewater such as domestic wastewater, dye wastewater and feed wastewater since it could provide removal of high organic load, resistance to organic shock load, odorless, easy operation and maintenance. The aeration in the system promoted growth of microorganisms and conferred uniform population which enhanced the consumption of organic loads (Hammer, 1991; Metcalf & Eddy, 2004). Accordingly, in this study, time course of growth and decolorization of bacterial consortium CONS8 was investigated in MM medium with the initial pH of 4 under aerobic conditions. In order to increase the bacterial biomass, the inoculum was prepared in LB medium prior to transfer into MM medium and cultivated under conditions as indicated above. A typical time course of molasses decolorization in MM medium by a bacterial consortium CONS8 under selected condition is shown in Fig. 4.8. The consortium notabely decolorized MM medium during the first 24 h of culture, and the color removal of 20% was observed after 48 h. The growth of the consortium slightly increased with the increase of cultivation period. In addition, the progression decolorization of molasses wastewater in MM medium by bacterial consortium CONS8 can be observed in the visible spectral sequence presented in Fig. 4.9. The absorbance from 400 to 700 nm decreased along the incubation time.

In this work, although the decolorization with the bacterial consortium CONS8 was lower than those of Basidiomycetes and Deuteromycetes previously reported (Friedrich, 2004; Gonzalez et al., 2000), it still possesses two advantages, i.e. the consortium showed high molasses decolorization under static conditions resulting in saving energy consumption during operation and application of fungi to remove melanoidins in molasses wastewater was a drawback due to their slow growth, spore production and infectivity (Friedrich, 2004). Bacterial decolorization of molasses

wastewater has been reported by many researchers (Dahiya et al., 2001; Murata et al., 1992; Nakajima-Kambe et al., 1999; Sirianuntapiboon et al., 2004a).

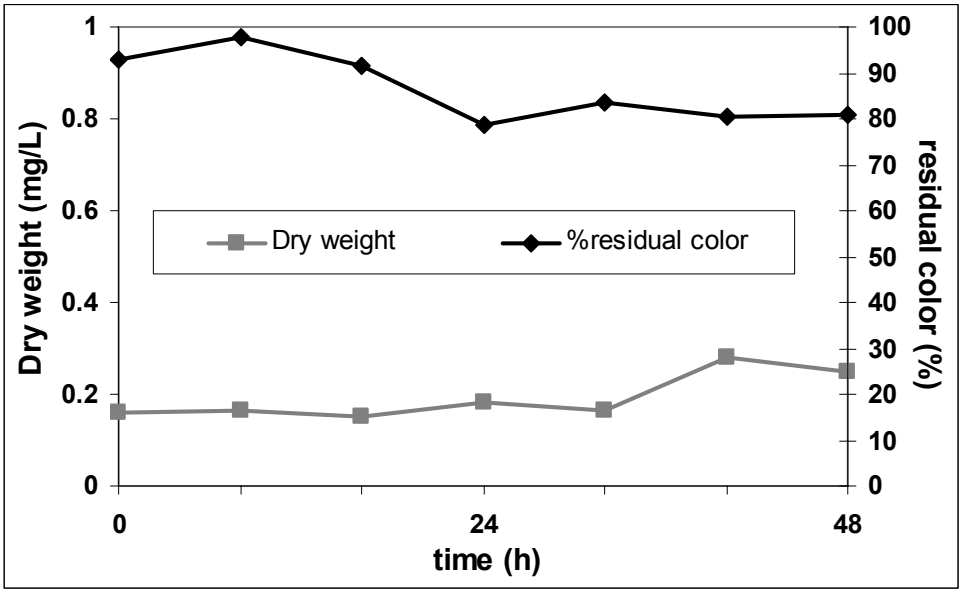


Figure 4.8 Decolorization of molasses wastewater by bacterial consortium CONS8 in MM medium at pH4 versus time. The consortium was grown under aerobic conditions.

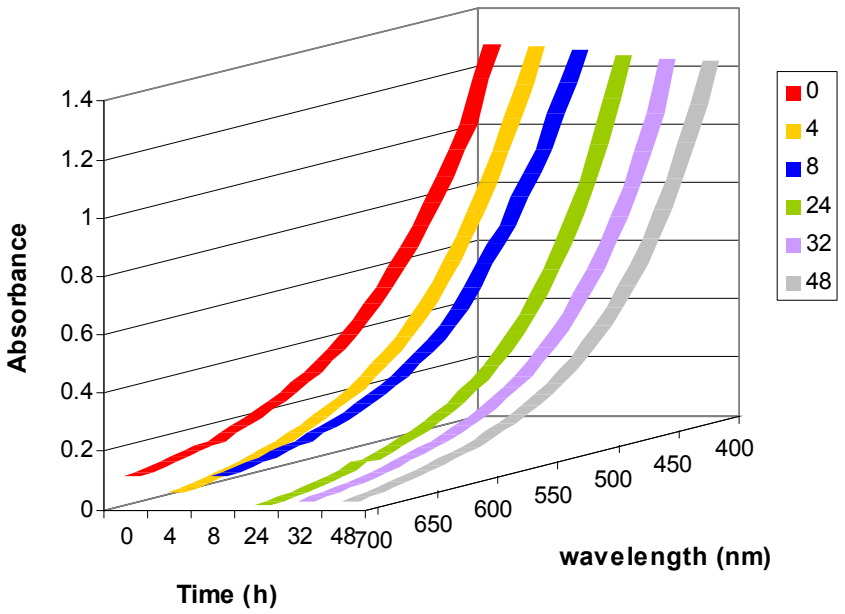


Figure 4.9 Typical visible spectra of supernatant from aerobic decolorization of sugarcane molasses wastewater by bacterial consortium CONS8 under the optimal conditions at various incubation times.

4.5 Identification of bacterial isolates present in the consortium CONS8

The molasses wastewater decolorizing-bacterial consortium CONS8, enriched in LB medium under aerobic conditions, was comprised of 8 (AE1-AE8) bacterial members from different colonies. Each kind of bacteria was identified by BLAST homology based on its partial 16S rDNA sequence as shown in Table 4.4. Some of bacterial strains present in the bacterial consortium CONS8 were previously reported as a molasses decolorizing bacteria. *Pseudomonas*, *Acinetobacter*, *Klebsiella*, and *Bacillus* could decolorize colored components present in molasses wastewater (Kumar and Chandra, 2006; Mohana et al., 2007; Petruccioli et al., 2000). However, the strains AE2 and AE5 had sequence homology of 16S rDNA with other genera deposited as unidentified strains in GENBANK.

Table 4.4 Percent similarity based on the alignments of the partial 16S rDNA sequences of isolated bacteria from consortium CONS8 to their closest bacterial relatives present in the NCBI nucleotide sequence database.

Bacterial strains	Accession no. of the closest strains	Sequences similarity	Closest strains
AE1	DQ226207	96%	*Serratia marcescens*
AE2	DQ816308	99%	Unknown bacterium
AE3	DQ226213	100%	*Acinetobacter* sp.
AE4	AY689030	98%	*Pseudomonas* sp.
AE5	DQ817737	98%	Unknown bacterium
AE6	AF188304	97%	*Comamonas* sp.
AE7	DQ226215	99%	*Klebsiella oxytoca*
AE8	AF529355	97%	Unknown gamma proteobacterium

4.6 Analysis of bacterial community

PCR-DGGE method based on 16S rDNA was used to assess changes in microbial communities (Jensen et al., 1998). In this study, the changes in microbial community were investigated among four culture media at the initial pH of 4 under aerobic conditions. DGGE patterns from bacterial consortium CONS8 with different media in aerobic condition were compared with those of pure cultures (Fig.4.10). Profiles of bacterial consortium CONS8 in LB medium (lane 1), LBWW medium (lane 2), MM medium (lane 3) and WW medium (lane 4) are comparable with a common band dominantly detected, potentially corresponding to AE3 (lane 7).

DGGE analyses showed significant difference in the bacterial community grown on the different media (Fig 4.10). In addition, DGGE analysis revealed that different bacterial strains were responsible for the molasses decolorization depending on the different culture conditions. Therefore, the selection of aeration condition and medium compositions were factors shaping the bacterial composition in the molasses wastewater-decolorizing consortium.

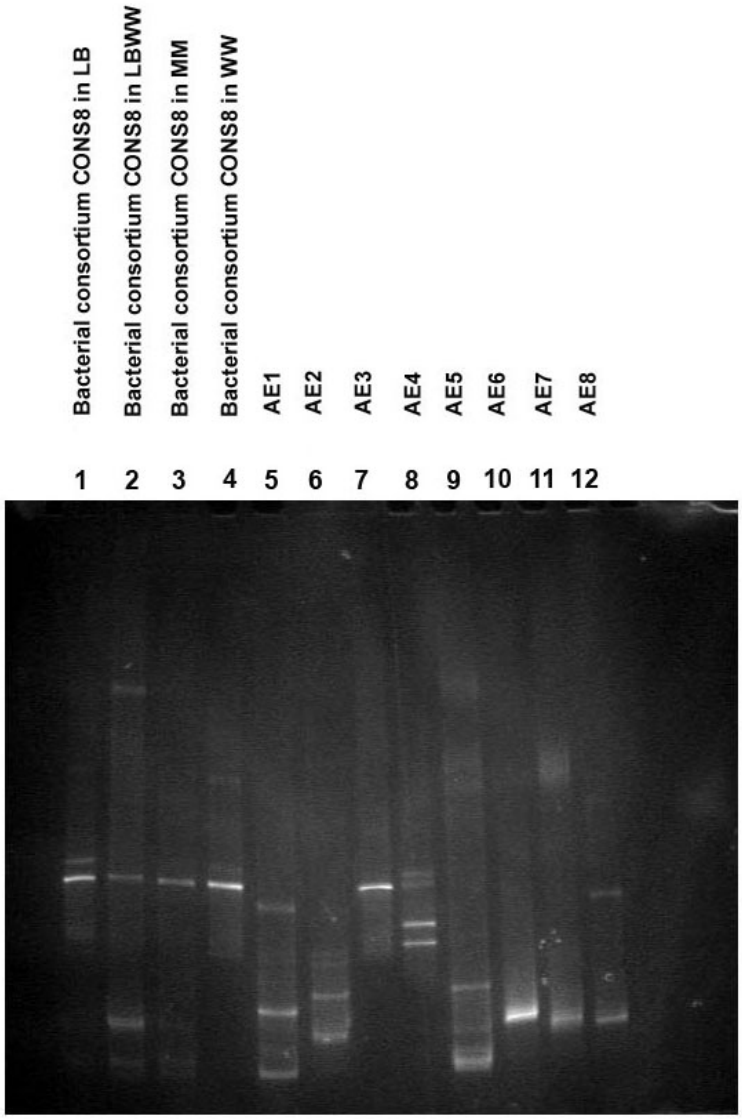


Figure 4.10 DGGE analysis of 16S rDNA sequences amplified from DNA extracted from culture of bacterial consortium CONS8 after enrichment in different culture media under aerobic conditions using PRBA338f and PRUN518r primers. Lanes: 1, Bacterial consortium CONS8 enriched in LB medium; 2, Bacterial consortium CONS8 enriched in LBWW medium; 3, Bacterial consortium CONS8 enriched in MM medium; 4, Bacterial consortium CONS8 enriched in WW medium; 5, AE1 (*Serratia marcescens)*; 6, AE2 *(* unknown bacterium); 7, AE3 (*Acinetobacter* sp.); 8, AE4 (*Pseudomonas* sp.); 9, AE5 (unknown bacterium); 10, AE6 (*Comamonas* sp.); 11, AE7 (*Klebsiella oxytoca*); and 12, AE8 (unknown gamma proteobacterium).

CHAPTER V

RESULTS
(ADJUSTMENT OF SOME OPERATING CONDITIONS PARAMETERS)

5.1 Choice of the synthetic substrate

Decolorization of various brown color substances by bacterial consortium CONS8

Generally, wastewaters obtained from sugarcane molasses based distilleries have no consistency and uniformity, as the compositions in wastewater such as COD, BOD, chemical elements and color substances vary depending on time, day or season (Mogan, 2002). For this study, the variations in real sugarcane molasses wastewater would affect the results of all experiments. Moreover, sugarcane molasses themselves contain varied amounts of melanoidins, dark brown to black colored natural condensation products of sugars, and amino acids produced by Maillard reactions, depending upon the nature of its source. Apart from melanoidins, sugarcane molasses also contain other colorants such as phenolic compounds and caramel, whereas melanin is abundant in beet molasses (Godshall, 1999). Therefore, in order to prevent the lack of raw sugarcane molasses wastewater supply as well as variations in its compositions, various kinds of synthetic melanoidins-containing wastewater media were formulated.

The aim of this study was to determine the decolorization capabilities of the bacterial consortium CONS8 in various kinds of synthetic melanoidins-containing wastewater media and to look for the most suitable synthetic melanoidins-containing wastewater to be used for further decolorization experiments. Various synthetic melanoidins-containing wastewater media were prepared according to the method mentioned previously in Chapter 3. In brief, three kinds of melanoidins-containing substances were used after dilution with distilled water to concentrations corresponding to color and COD contents of raw sugarcane molasses wastewater. Then, the bacterial consortium CONS8 was grown at 30°C to investigate the decolorization of its model brown color substances. The characteristics of individual melanoidins color substances were indicated in Table 5.1 and the spectrophotometric determination of each melanoidins color substances is shown in Fig. 5.1.

Table 5.1 Characteristics of synthetic melanoidins-containing wastewater media

Color substances	Initial concentration % (v/v)	OD_{475}	COD (g/L)
Viandox sauce	13.5	5.7081	22.8
Beet molasses wastewater	41.5	5.9014	30.75
Sugarcane molasses wastewater	20	5.7091	21.6

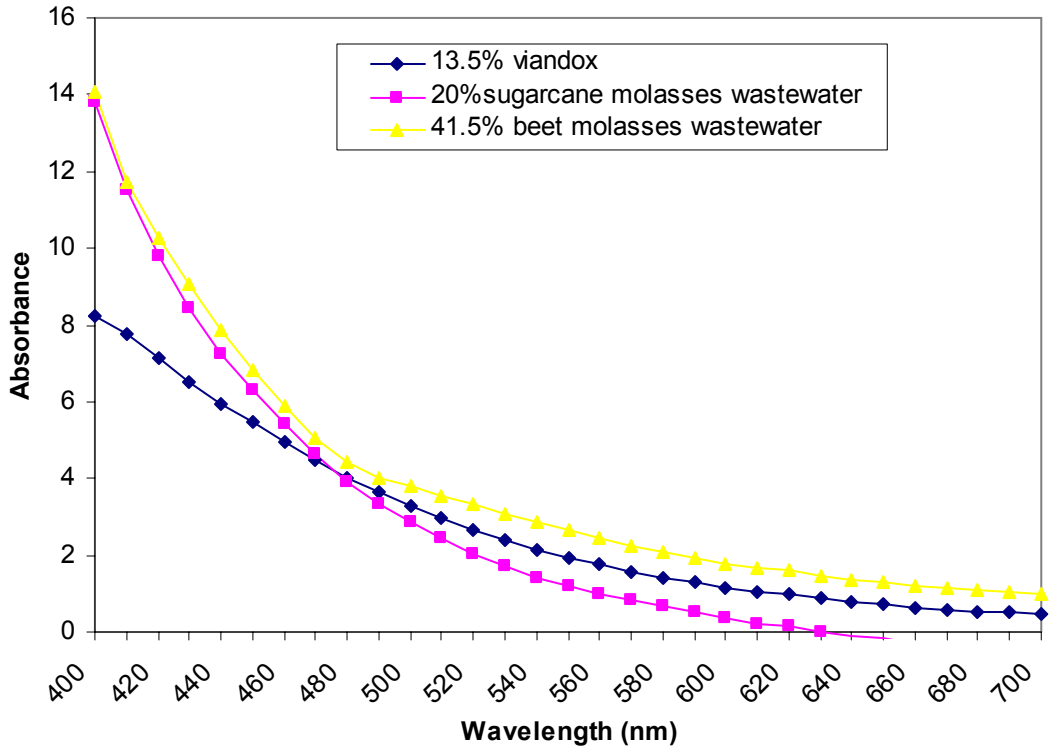


Figure 5.1 Typical visible spectrum of the individual synthetic melanoidins-containing wastewater medium before incubation of bacterial consortium CONS8.

The sugarcane molasses decolorization of bacterial consortium CONS8 with Viandox sauce, and beet molasses wastewater is given in Fig. 5.2. It shows that the decolorization of 17.5%, 9.5%, and 8.02% were achieved when using sugarcane molasses wastewater, Viandox sauce and beet molasses wastewater as a color substance of synthetic wastewater, after aerobic incubation for 48 h, respectively. The decolorization of the bacterial consortium CONS8 differed with the model color substances. The consortium could decolorize the model melanoidins pigments presented in sugarcane molasses wastewater, beet molasses wastewater and Viandox.

Figure 5.3 shows that bacterial growth was achieved when using real sugarcane molasses wastewater from Thailand, Viandox sauce, and beet molasses

wastewater as brown colored substances in the synthetic melanoidins-containing wastewater.

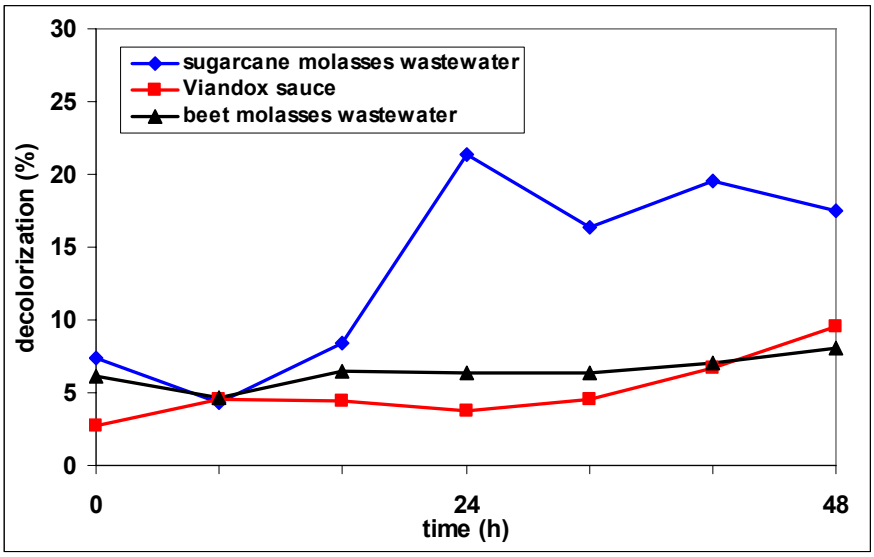


Figure 5.2 Decolorization of various synthetic melanoidins-containing wastewaters by the bacterial consortium CONS8. The data were obtained from three independent experiments.

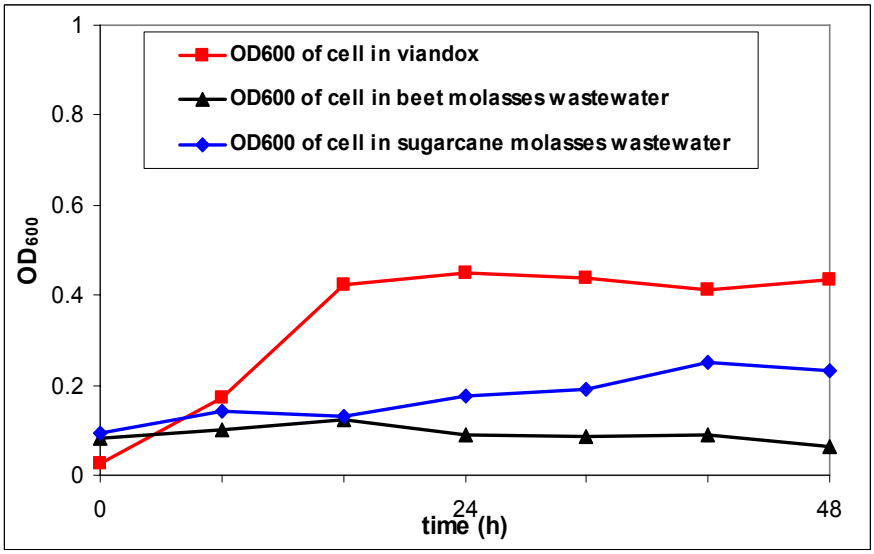


Figure 5.3 Growth of the bacterial consortium in various synthetic melanoidins-containing wastewaters. The data were obtained from three independent experiments.

It appeared that the Viandox sauce seemed to be the best source of brown colored substances in the model sugarcane molasses based distillery effluent. Then, the Viandox sauce will replace the real sugarcane molasses wastewater from Thailand since the variation of its compositions is rather lower than real sugarcane molasses wastewater. Therefore, Viandox sauce was used as the only colored substrate in the synthetic melanoidins-containing wastewater medium which will be used for all further experiments. However, the initial chosen concentration of Viandox at 13.5% (v/v) was not suitable as a colored substance in synthetic melanoidins-wastewater because this concentration could interrupt the bacterial growth (partial inhibition). Hence, the initial concentration of Viandox at 2% (v/v) was selected for further study.

In this study, another experiment was carried out to determine the variety of melanoidins decolorization of the bacterial consortium CONS8. Melanoidins models were also prepared in the laboratory using glucose and glycine as sugar and amino acid precursor for Maillard reaction (Dahiya et al., 2001). A mixture of 1 M glucose, 1 M glycine and 0.5 M sodium carbonate was autoclaved at 121°C for 15 min. The solution was adjusted to pH 7.0 with 1 N NaOH. The dark brown product, which assumed as the synthetic glucose/glycine model melanoidins, was used as a color substrate in the synthetic melanoidins-containing wastewater consisted of 0.01% $NaNO_3$, 0.2% K_2HPO_4, 0.1% KH_2PO_4, 0.01% $MgSO_4 \cdot 12H_2O$, 2% glucose and 0.1% yeast extract. The initial optical density (color) of medium at 475 nm was adjusted to the value of 3.5 by addition of synthetic melanoidins solution. The bacterial consortium CONS8 was investigated for its model melanoidins decolorization using the agar plate method and the shaking culture method as previously described in Chapter 3.

The results from both methods indicate that the bacterial consortium CONS8 did not show any decolorization against the glucose-glycine melanoidins model and the bacterial biomass content rather decreased along the incubation time like. This result might be due to the high inhibitory and antimicrobial activity of prepared melanoidins which could inhibit the growth and decolorization of bacterial consortium CONS8. This result is similar to those reported previously by many authors (Taylor et al., 2004; Rufian-Henares and Morales, 2006; Painter, 1998; Hiramoto et al., 1997).

It has been reported that melanoidins have negative biological effects such as mutagenic, carcinogenic and cytotoxic effects and give high inhibitory and antimicrobial activity (Painter, 1998; Taylor et al., 2001). Some harmful biological effects of melanoidins have been reported as followed: destruction of essential amino acids, inactivation of enzymes, inhibition of regulatory molecule binding, cross-linking

of glycated extracellular matrix, abnormalities of nucleic acid function, altered macromolecular recognition, endocytosis and increased immunogenicity. Mutagenicity and DNA strand breaking activity of melanoidins from a glucose–glycine model was demonstrated by Hiramoto et al. (1997) who reported that the low molecular weight fractions acted as lipid sink (Larter and Douglas, 1980) and induced DNA damage, where the effect increased with the concentration added.

5.2 Effect of initial pH on decolorization of synthetic melanoidins-containing wastewater

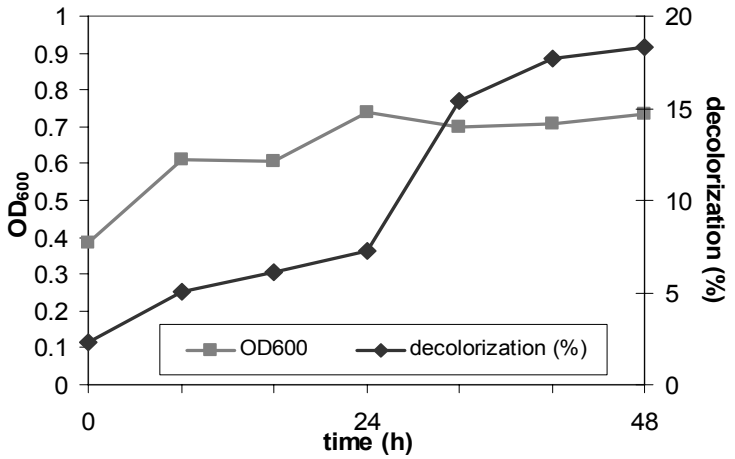
Environmental factors like pH, colored substances, aeration and nutrients play vital role in bacterial decolorization of molasses based distilleries as the metabolism and activity of enzymes are greatly influenced by these environmental factors. In order to characterize the pH influence for the effective decolorization by bacterial consortium CONS8, experiments were performed at three different initial pH values (4, 7 and 9). The effect of initial pH on the decolorization and growth profiles of bacterial consortium were given in Figure 5.3. The bacterial consortium could grow and decolorize synthetic melanoidins-containing wastewater made of 2% (v/v) Viandox at both pH 4 and pH 7 (Figure. 5.4a and 5.4b, respectively). However, this bacterial consortium led to the much lower decolorization at pH 9 (Fig. 5.4c). The highest decolorization of bacterial consortium was observed with an initial medium pH of 4 whereas; the decolorization was decreased when the initial pH of medium was higher than 7 or highly alkaline.

It has been demonstrated previously that the initial acidic pH has a critical effect on melanoidins decolorization. The similar effect was also observed in another study where optimal decolorization of sugarcane molasses wastewater by soil inoculum was obtained at acidic pH (Alkane et al., 2006). Alkane et al. (2006) reported that pH has a crucial role in melanoidins decolorization. An increase in pH of medium resulted in less microbial decolorization and the increase in color intensity. The increase in color may be due to the polymerization of melanoidins (Alkane et al., 2006). The decrease in color removal efficiency in highly alkaline pH might be due to the fact that the melanoidins responsible for color were more soluble in the alkaline pH, whereas, the melanoidins might be precipitated and removed easily in the acidic pH.

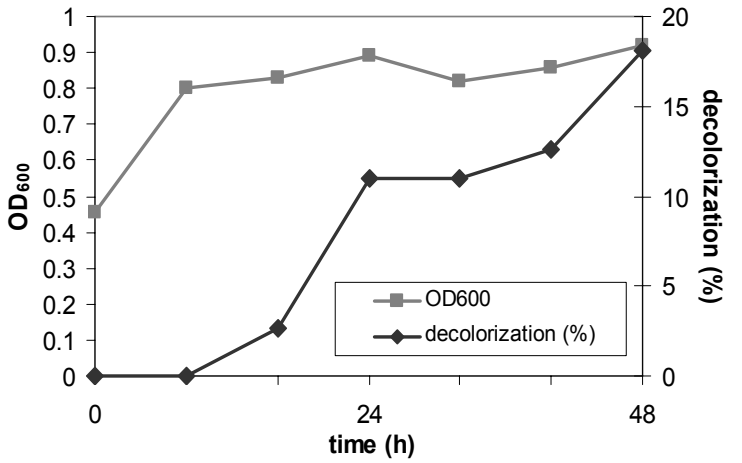
Therefore, initial acidic pH seems to favor the decolorization of this bacterial consortium. Indeed, pH 4 is closed to pH value of sugarcane molasses wastewater in Thailand. Hence, synthetic melanoidins-containing wastewater containing 2% (v/v)

Viandox at initial pH 4 was selected for further studies since this condition gave the maximal decolorization of 18.3% under aerobic condition for 48 h (Fig. 5.4a). In addition, the progressive decolorization of synthetic melanoidins-containing wastewater medium containing 2% (v/v) Viandox as a colored substrate at the initial pH 4 by bacterial consortium CONS8 can be observed in the visible spectral sequence presented in Fig. 5.5. The absorbance from 400 to 700 nm decreased along the incubation time.

(a)



(b)



(c)

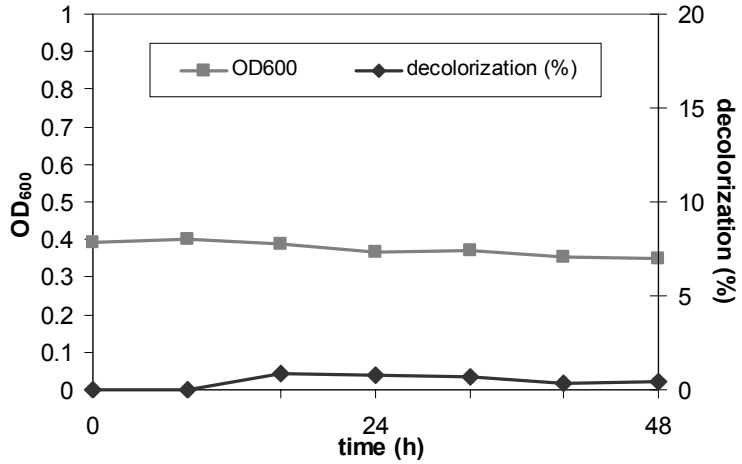


Figure 5.4 Time course of growth and decolorization of bacterial consortium using synthetic melanoidins-containing wastewater medium containing 2% (v/v) Viandox as a colored substrate at the initial pH 4 (a); pH 7 (b) and pH 9 (c).

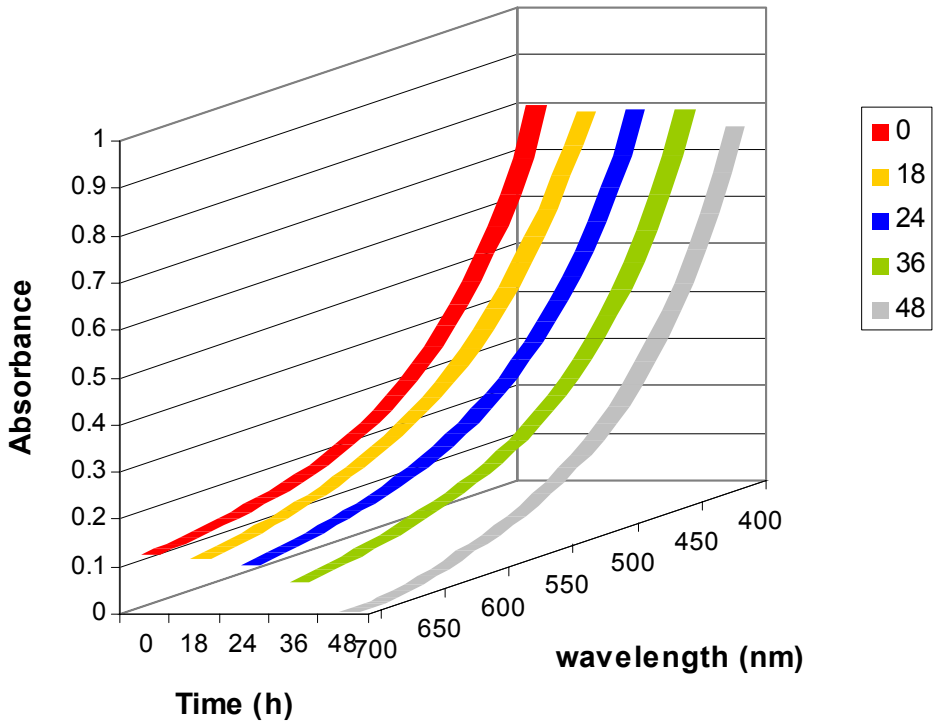


Figure 5.5 Typical visible spectra of supernatant from aerobic decolorization of synthetic melanoidins-containing wastewater medium containing 2% (v/v) Viandox as a colored substrate at the initial pH 4 by bacterial consortium CONS8 under the optimum condition at various incubation times.

CHAPTER VI

RESULTS
(CONSTRUCTION OF ARTIFICIALLY STRUCTURED BACTERIAL CONSORTIUM)

6.1 Identification of bacterial isolates in the consortium contributing melanoidins decolorization

The previous experiments on the organization of bacterial consortium CONS8 in the Chapter 5 were done with of natural composition and architecture. Bacterial consortia in nature are typically formed by bacterial strains, normally heterogeneous, and often have complex structures. The organization of these consortia can be expected to have a significant influence on the function of the cells within the bacterial consortia. The ability to create new consortium with defined architecture, with different strains of bacteria, could result in a significant advance in the methods available for the optimization of bacterial decolorization and might offer a good strategy for obtaining high decolorization.

Hence, in the present experiments, the study was focused on the construction of a high potential melanoidins decolorizing bacterial consortium. The aim was to investigate the possibility of constructing artificially structured bacterial consortium for decolorization of the synthetic melanoidins-containing wastewater medium.

A prerequisite for this study was the ability to separate species on selective media to facilitate analysis. Serial subcultures of the consortium CONS8 were carried out on melanoidins-containing wastewater medium to discriminate the effective melanoidins-decolorizing species.

Subsequently, the decolorizing bacterial isolates present after 5 successive subcultures were isolated to pure culture by morphological difference. This was performed by examining growth, colony size, color, and morphology on LB medium agar plates after incubation for 24 h and confirmed by Gram staining. The different individual bacterial colonies on LB agar plates are shown in Figure 6.1.

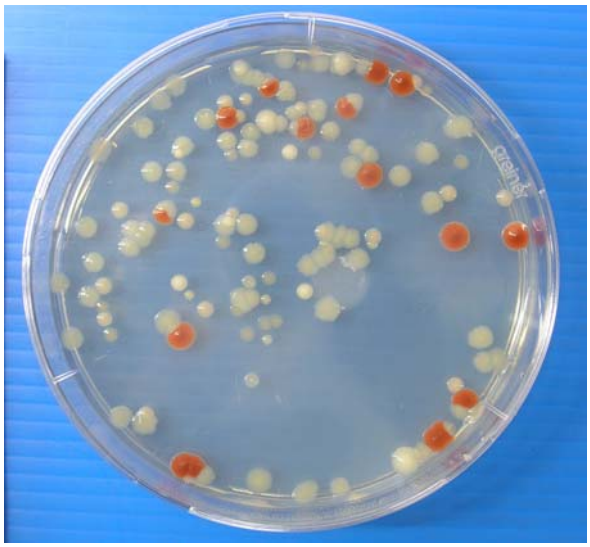


Figure 6.1 View of the various bacterial colonies on LB agar plate after 5 successive subcultures of consortium CONS8 under aerobic conditions.

The individual bacterial isolates shown in Figure 6.1 were grown in LB agar plate under aerobic conditions for 24 h at 30^{O}C. Each isolate in the consortium CONS8 was further identified by partial 16S rDNA sequence. The 16S rDNA gene PCR amplified from the isolates using 20F and 802R primers was subjected to DNA sequencing with UFUL internal primers (Nilsson et al., 2003). The sequences were then compared using NCBI BLASTN program. Pairwise alignments giving a closest match of 99% or more were chosen.

The enriched consortium CONS8 mainly comprised of *Klebsiella oxytoca* (T1), *Serratia mercescens* (T2), *Citrobacter farmeri* (T3) and unknown bacterium DQ817737 (T4). The 16S rDNA sequences of these bacteria were shown in Appendix 2.

Some of these species have been reported as molasses decolorizing bacteria. They were *Serratia mercescens*, *Citrobacter* sp and *Klebsiella* present in this bacterial consortium. All of them could decolorize colored components present in molasses wastewater (Kumar and Chandra R, 2006; Mohana et al., 2007; Petruccioli, 2000). Many researchers also reported the activity of *Serratia mercescens* on biodegradation of polycyclic aromatic hydrocarbons (PAHs) and lignin degrading activity (Rhoads et al., 1995).

6.2 Construction **of artificially structured bacterial** consortia

To verify whether the decolorization of melanoidins-containing wastewater is more effective by artificially constructed bacterial consortia than either consortium CONS8 or individual bacterial strains, the experiment was carried out by constructing different bacterial consortia. Factorial experimental design was applied to identify the potent melanoidins-decolorizing bacterial isolates and to determine the efficient bacterial compositions.

The four predominant bacterial members present in the consortium CONS8, *Klebsiella oxytoc*a (T1), *Serratia mercescens* (T2), *Citrobacter farmeri* (T3) and unknown bacterium (DQ817737) (T4) were chosen as independent variables and the percentage of decolorization was the dependent response variable.16 different experiments were carried out according to the factorial method for 4 bacteria (T1-T4) in comparison with control (Table 6.1.).

Table 6.1 Experimental design for decolorization of the synthetic melanoidins-containing wastewater medium with the four isolates

Experiments	Bacterial composition
1	T1
2	T2
3	T3
4	T4
5	T1+T2
6	T1+T3
7	T1+T4
8	T2+T3
9	T2+T4
10	T3+T4
11	T1+T2+T3
12	T1+T2+T4
13	T1+T3+T4
14	T2+T3+T4
15	T1+T2+T3+T4
control	No bacterium

Note: T1= *Serratia mercescens*, T2= *Klebsiella oxytoca*, T3= *Citrobacter farmeri* and T4= unknown bacterium DQ817737

The summarized results of decolorization of the synthetic melanoidins-containing wastewater by individual strains and by different mixture of strains after incubation under aerobic conditions for 72 h are shown in Figure 6.2. The decolorization varied among experiments . The bacterial growth is also presented in Figures 6.3 – 6.18. They showed that the effectiveness of decolorization depends on bacterial components of the inoculum. The results indicated that the decolorization varied significantly in the range of 0 – 17.5% with the bacterial composition tested.

Individually, unknown bacterium (DQ817737) (T4), *Klebsiella oxytoc*a (T1) and *Citrobacter farmeri* (T3) could decolorize melanoidins-containing wastewater up to 11.4%, 8.94% and 6.72% in 72 h, respectively, under aerobic conditions (Figure 6.2). The results show that unknown bacterium DQ817737 (T4) accounted for the

highest of decolorization of synthetic melanoidins-containing wastewater. Lowest decolorization was observed when pure culture of *Serratia mercescens* (T2) was used as inoculum (experiment 2).

Although *Serratia mercescens* (T2) was not an effective decolorizer, its presence might still play an important role in increasing color removals of various bacterial consortia (experiment 5, 8, 11, 12, 14 and 15). It seems that decolorization activities in these experiments was enhanced by the presence of *Serratia mercescens* (T2).

The experimental results also illustrated that the bacterial consortia were able to decolorize synthetic melanoidins-containing wastewater at higher level as compared to those achieved by individual isolates. Decolorization values above 15% were only observed when consortia were composed of strain T1, T2 and T4 together (experiment 12 and 15). The experimental results suggest that the bacterial composition obviously affect the decolorization.

The result in Figure 6.3 shows that the decolorization did not occur in sterile cell-free medium, suggesting the absence of abiotic decolorization.

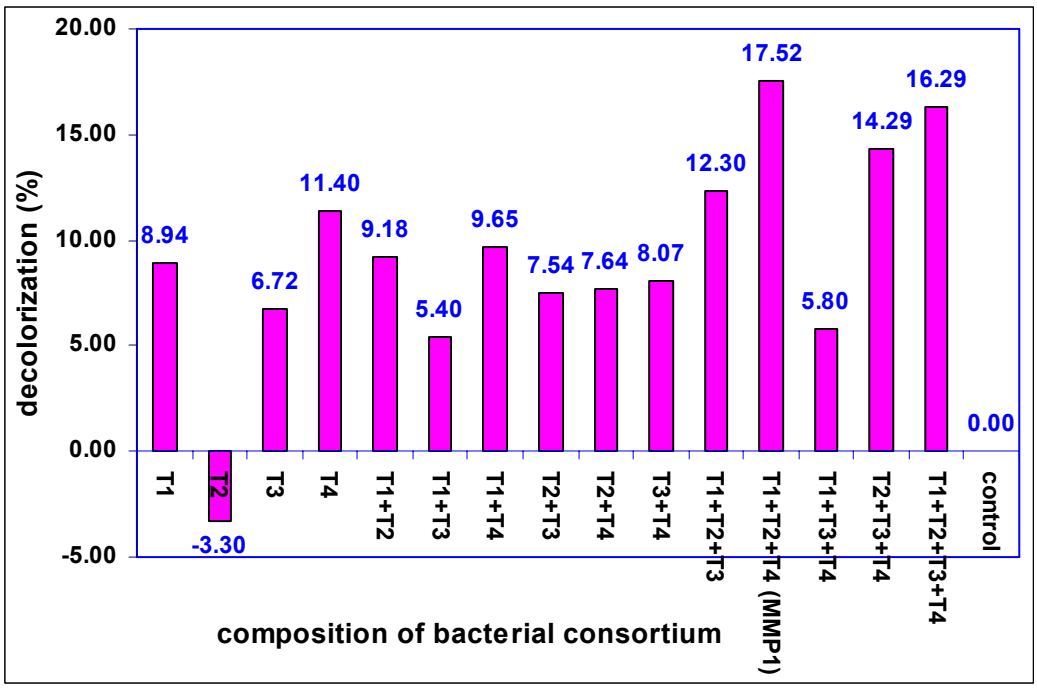


Figure 6.2 Percent decolorization of the synthetic melanoidins-containing wastewater medium containing 2% (v/v) Viandox by different bacterial consortia. Negative value indicates the increase in color; the data result of three independent experiments.

As might be expected for very simple bacterial consortia, these results suggested that *Klebsiella oxytoca* (T1), *Serratia mercescens* (T2), and unknown bacterium (DQ817737) (T4) strongly contributed to the decolorization of melanoidins in many consortia as shown in experiment 5, 8, 11, 12, 14 and 15.

As shown in Figure 6.2, a **consortium MMP1 comprising** of three bacteria; *Klebsiella oxytoca* (T1), *Serratia mercescens* (T2), and unknown bacterium (DQ817737) (T4) exhibited the same degree of decolorization to the mixed population with the four **bacteria (experiment 15).** Moreover, the degree of decolorization (17.5 %) of the melanoidins by the consortium MMP1 was almost equal to those of the bacterial consortium CONS8 with natural architectures under the same culture conditions (Figure 5.4a). Thus, bacterial consortium MMP1 appeared to be a good choice rather than consortium CONS8 in term of prolonging the high decolorization efficiency and suitable for in situ application.

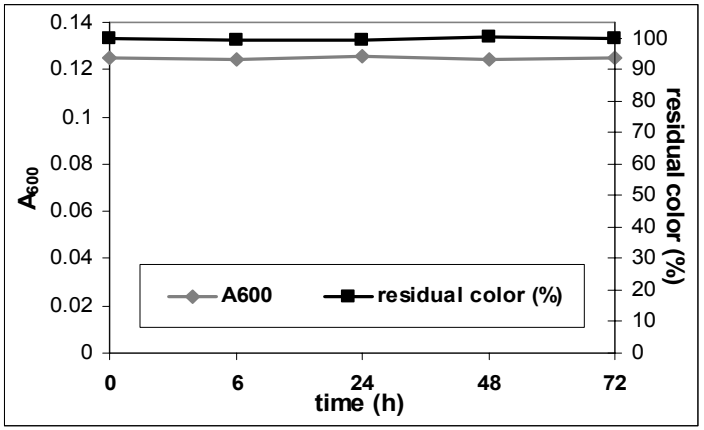


Figure 6.3 Time course of growth and decolorization of the control experiment (without bacteria)

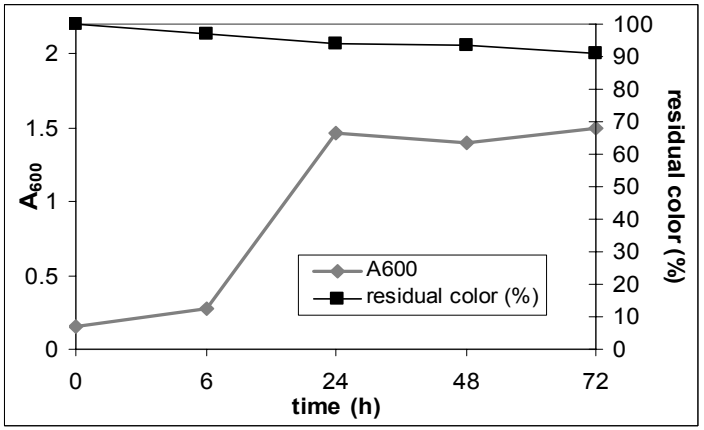


Figure 6.4 Time course of growth and decolorization of the experiment 1 (culture T1)

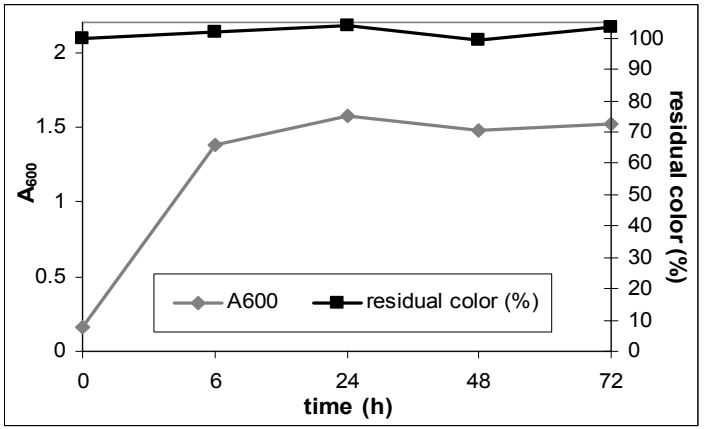


Figure 6.5 Time course of growth and decolorization of the experiment 2 (culture T2)

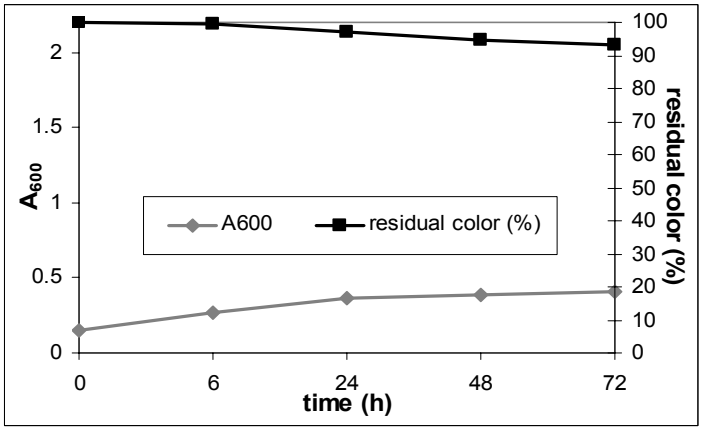


Figure 6.6 Time course of growth and decolorization of the experiment 3 (culture T3)

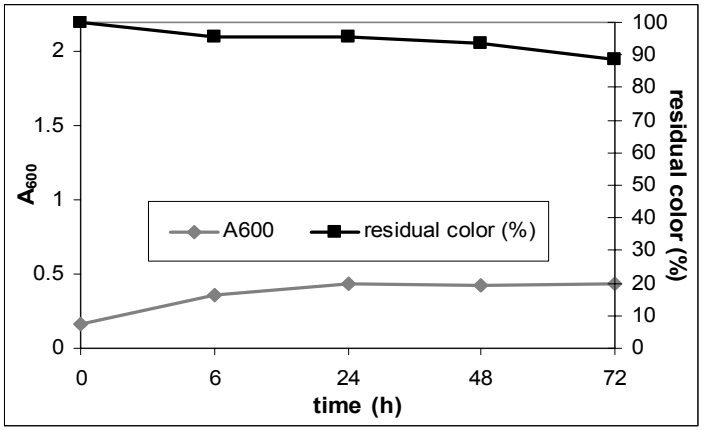


Figure 6.7 Time course of growth and decolorization of the experiment 4 (culture T4)

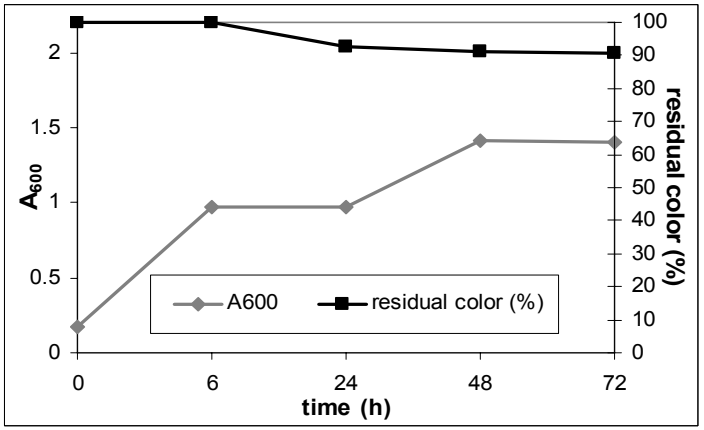


Figure 6.8 Time course of growth and decolorization of the experiment 5 (co-culture T1+T2)

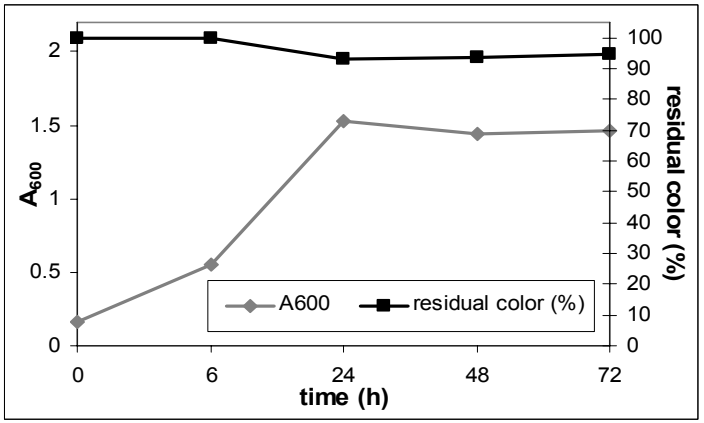


Figure 6.9 Time course of growth and decolorization of the experiment 6 (co-culture T1+T3)

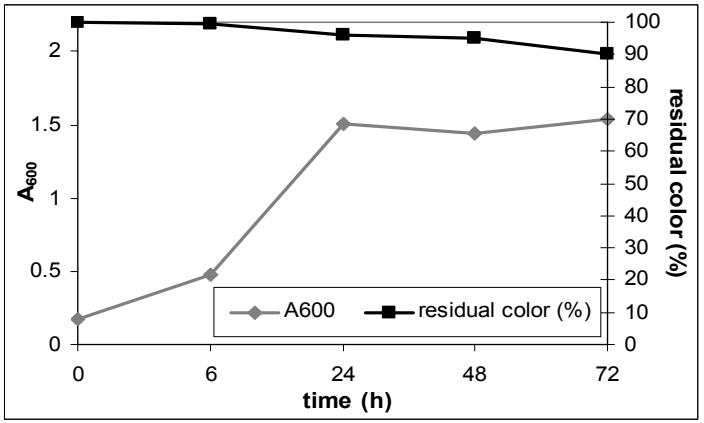


Figure 6.10 Time course of growth and decolorization of the experiment 7 (co-culture T1+T4)

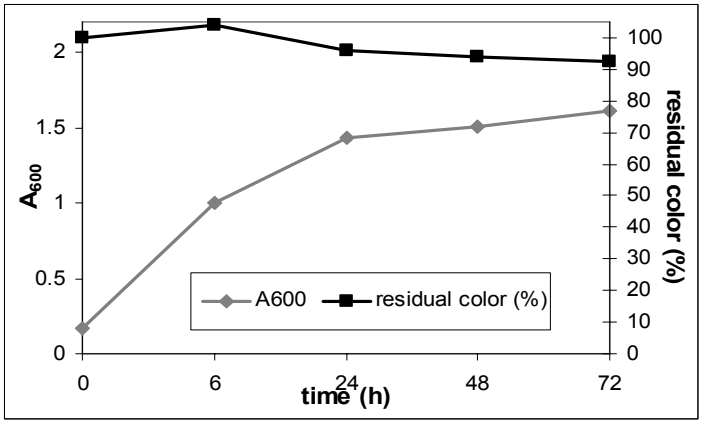


Figure 6.11 Time course of growth and decolorization of the experiment 8 (co-culture T2+T3)

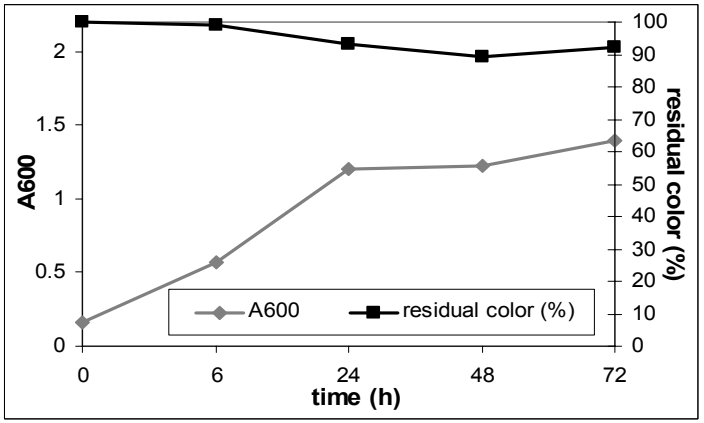


Figure 6.12 Time course of growth and decolorization of the experiment 9 (co-culture T2+T4)

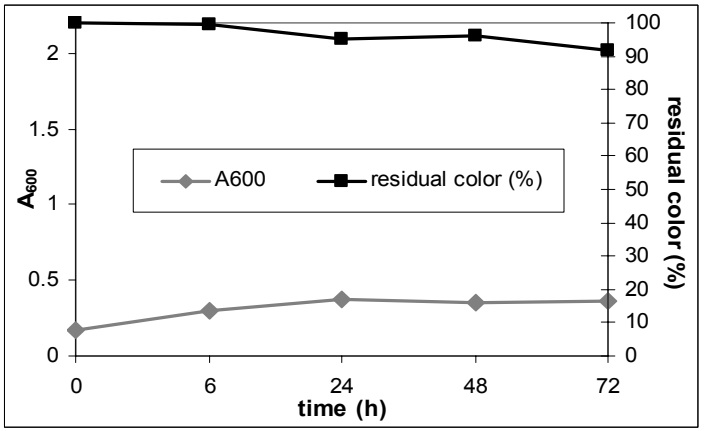


Figure 6.13 Time course of growth and decolorization of the experiment 10 (co-culture T3+T4)

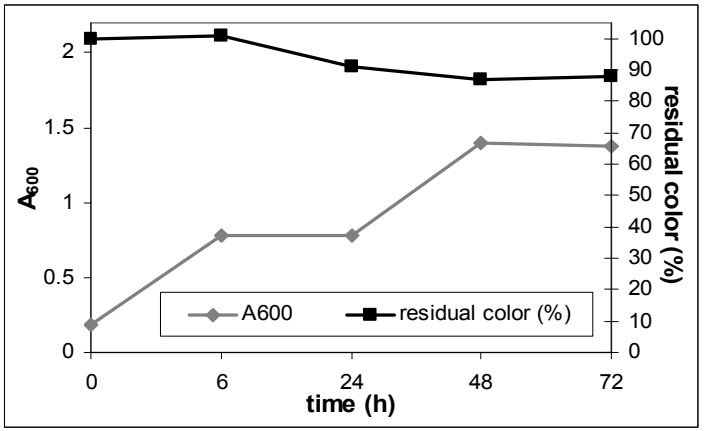


Figure 6.14 Time course of growth and decolorization of the experiment 11 (co-culture T1+T2+T3)

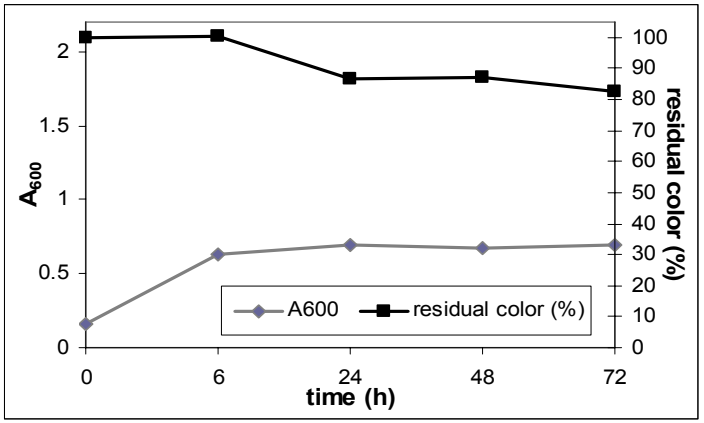


Figure 6.15 Time course of growth and decolorization of the experiment 12 (co-culture T1+T2+T4)

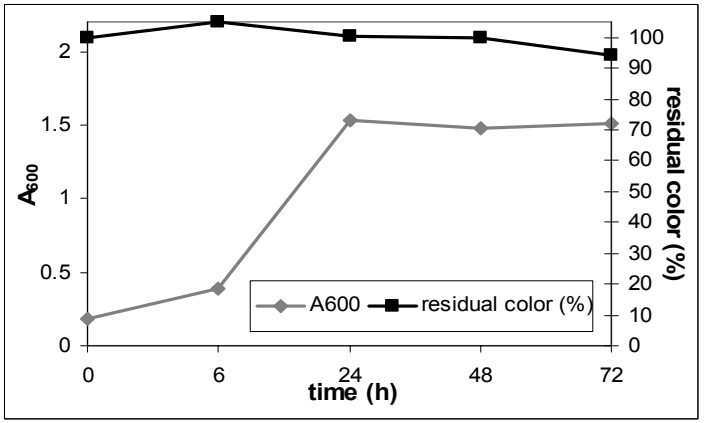


Figure 6.16 Time course of growth and decolorization of the experiment 13 (co-culture T1+T3+T4)

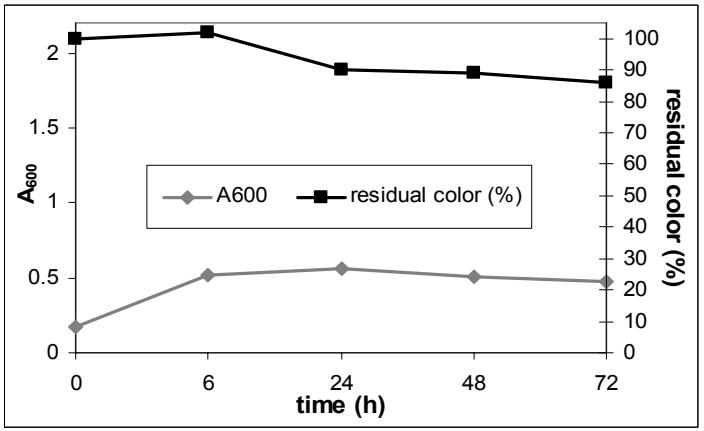


Figure 6.17 Time course of growth and decolorization of the experiment 14 (co-culture T2+T3+T4)

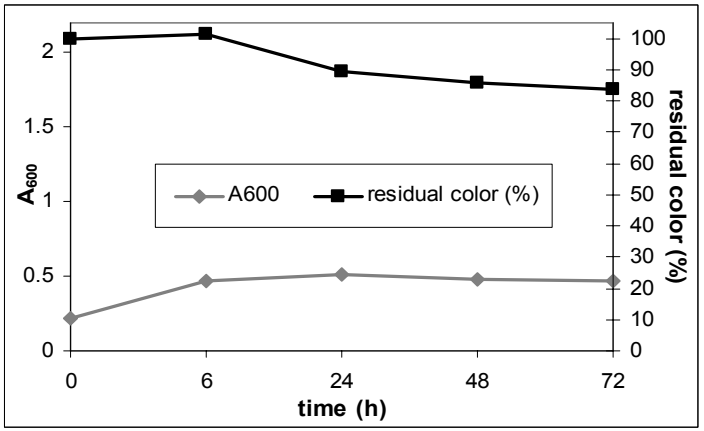


Figure 6.18 Time course of growth and decolorization of the experiment 15 (co-culture T1+T2+T3+T4)

In this experiment, the bacterial consortium namely MMP1, comprising of *Klebsiella oxytoca* (T1), *Serratia mercescens* (T2), and unknown bacterium DQ817737 (T4), was tested in further study due to its highest decolorization level 17.52%. Since the melanoidin decolorization process is enzyme dependent, the consortium showed synergistic effect and adapted metabolism to produce nontoxic metabolites. Furthermore, bacterial decolorization may require a mixed culture to decolorize molasses wastewater through combined metabolic mode of individual bacterial strain. Hence, the higher decolorization efficiency of consortium MMP1 may be due to the enhanced effect of coordinated metabolic interactions on melanoidins decolorization (Manjinder et al., 2005; Sarayu et al., 2006; ; Sarayu et al., 2008).

A similar pattern was also observed on the decolorization of bacterial consortium DMC, comprising of *Pseudomonas aeruginosa* PAO1, *Stenotrophomonas maltophila* and *Proteus mirabilis*, which achieved its maximum molasses decolorization activity 67% and 51% COD reduction within 72 h in the presence of 0.5% glucose (Mohana et al., 2007).

Similarly, Nigam et al. (1996) reported that although the individual bacterial isolates present in the consortium-PDW were unable to decolorize any dyes, however, the mixed bacterial culture-PDW was able to decolorize various textile dyes with a rather slow decolorization rate.

Senan and Abraham (2004) also developed a consortium of three bacteria to degrade a mixture of the dyes by co-metabolism and observed that the consortium could decolorize efficiently all the three dyes tested.

Hence, mixed bacterial culture seems to be more promising for molasses wastewater decolorization. This is due to the synergism present in mixed communities, as the catabolic activities of bacteria in the consortium complement each other.

6.3 Optimum decolorization condition of constructed bacterial consortium MMP1

To elucidate the cooperative action of mixed populations in the decolorization of melanoidins-containing wastewater, the artificial bacterial consortium MMP1 composed of three bacteria was used. Experiments were repeated to validate the optimum condition of bacterial strains in consortium MMP1. The decolorization efficiency of bacterial consortium MMP1 was investigated under the optimum condition. Precultures of *Serratia mercescens*, *Klebsiella oxytoca*, and unknown bacterium (DQ817737) were prepared in the synthetic melanoidins-containing wastewater medium. Decolorization of melanoidins by bacterial consortium MMP1

comprised of these three strains was examined in synthetic melanoidins-containing wastewater medium containing a Viandox sauce (2%, v/v) as a color substrate with the initial pH 4 at 30^{O}C under aerobic conditions for 72 h.

Figure 6.19 shows a typical culture profile of the constructed bacterial consortium MMP1. The plots of percentage decolorization (Figure 6.19) revealed the profile of decolorization with respect to changes in bacterial biomass, total nitrogen concentration and pH. These culture conditions resulted in approximately of 22% decolorization of Viandox sauce within 72 h. Further incubation did not enhance the decolorization. The total nitrogen decreased from 390 mg/L at the beginning of this experiment to 290 mg/L after incubation for 10 h and remained unchanged until 72h. The pH of cultured medium showed no significantly change during the overtime (Figure 6.19).

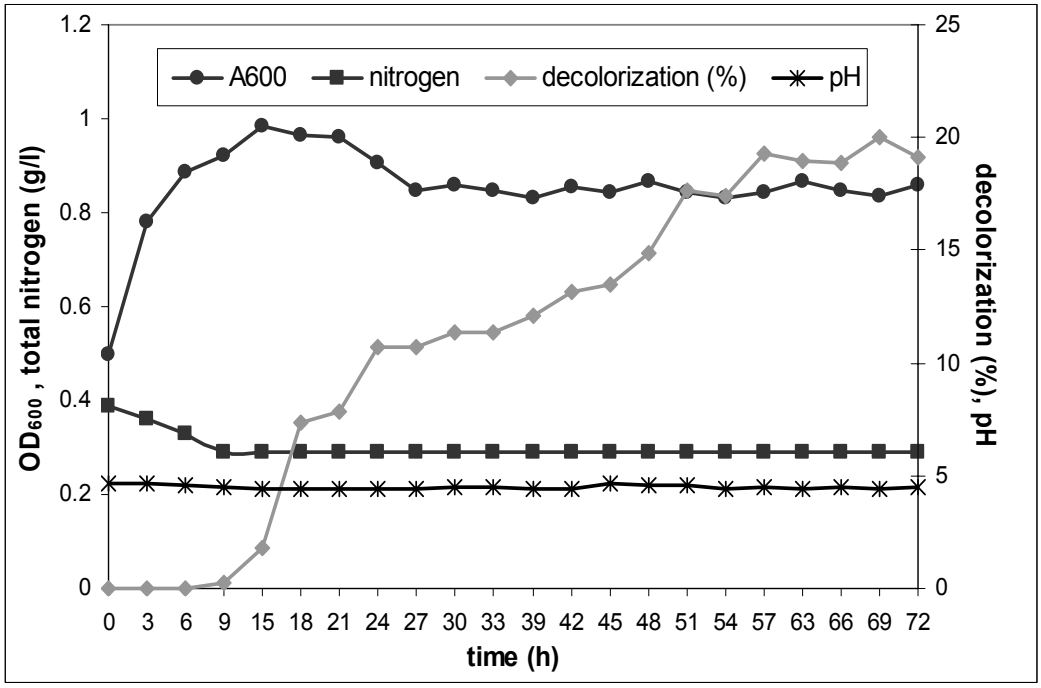


Figure 6.19 Growth and decolorization of melanoidins-containing wastewater by the constructed bacterial consortium MMP1 under optimal conditions.

In this chapter, the results indicated that the bacterial consortium MMP1 was able to decolorize synthetic melanoidins-containing wastewater. The optimum combination of the bacterial members (T1, T2, and T4) in the bacterial consortium MMP1 was confirmed through experiments and hence this constructed bacterial consortium holds a potential for the treatment of melanoidins-containing wastewaters. Application of the artificially constructed bacterial consortium MMP1, comprising *Serratia mercescens*, *Klebsiella oxytoca*, and unknown bacterium (DQ817737) to decolorization of melanoidins-containing wastewater seems to be a

pragmatic approach. Thus, further studies were carried out by using the artificial constructed bacterial consortium MMP1.

6.4 Optimum aeration condition of constructed bacterial consortium MMP1 in bioreactor

Aeration basically serves the purpose of providing the air required for biodegradation and keeping the biomass dispersed throughout the reactor. To investigate the effect of aeration on decolorization of synthetic melanoidins-containing wastewater medium by bacterial consortium MMP1, the experiment was carried out in a 3L laboratory-scale completely stirred tank reactor contained 2L of solution (BioFlo® 110 Fermentor & Bioreactor; New Brunswick Scientific Co., Inc. USA). The experimental set up used in this study is shown in Figure 6.20. The air was supplied through the reactor liquid phase using an air sparger at the bottom. DO concentration in the reactor was measured by a CO_2/O_2 analyzer (Xentra 4100 Gas Purify Analyser; Servomex S.A. France) (Figure 6.21). The oxygen concentration was varied at 0, 0.1, 0.2, and 0.4 vvm during the start-up by subsequent adjustment of oxygen loading by gas flowmeter. The calculation of K_La values at various aeration rates was shown in Appendix 3. The oxygen transfer coefficient (K_La) in the synthetic melanoidins-containing wastewater at various aeration rates was shown in Figure 6.22. Full mixing within the reactor was achieved with one set of mechanical stirrer, which was at the bottom of the liquid phase. The agitation speed was set at 150 rpm. Reactor was equipped with heat jackets in order to maintain an operational temperature of $30^{O}C$. The influence of DO on melanoidin decolorization by the bacterial consortium in bioreactor was investigated after the seed bacterial inoculation. Samples were taken for measurements of optical density at 600 and 475 nm.

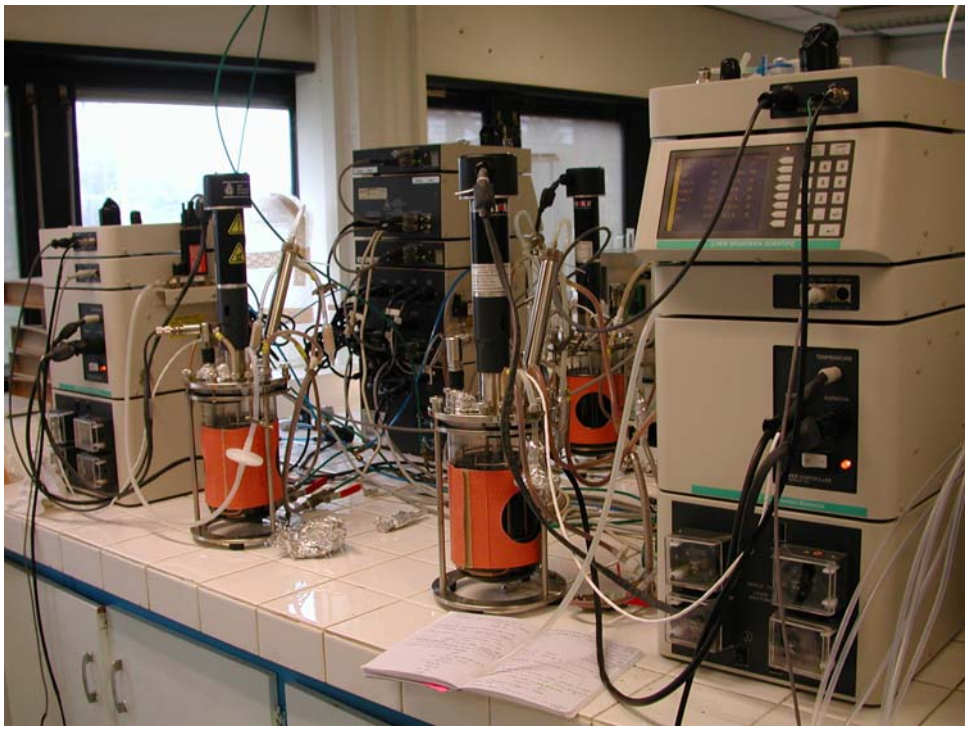


Figure 6.20 Laboratory-scale suspended cell bioreactor**.** (BioFlo® 110 Fermentor & Bioreactor; New Brunswick Scientific Co., Inc. USA)

Figure 6.21 CO_2/O_2 analyzer used in this study**.** (Xentra 4100 Gas Purify Analyser; Servomex S.A. France)

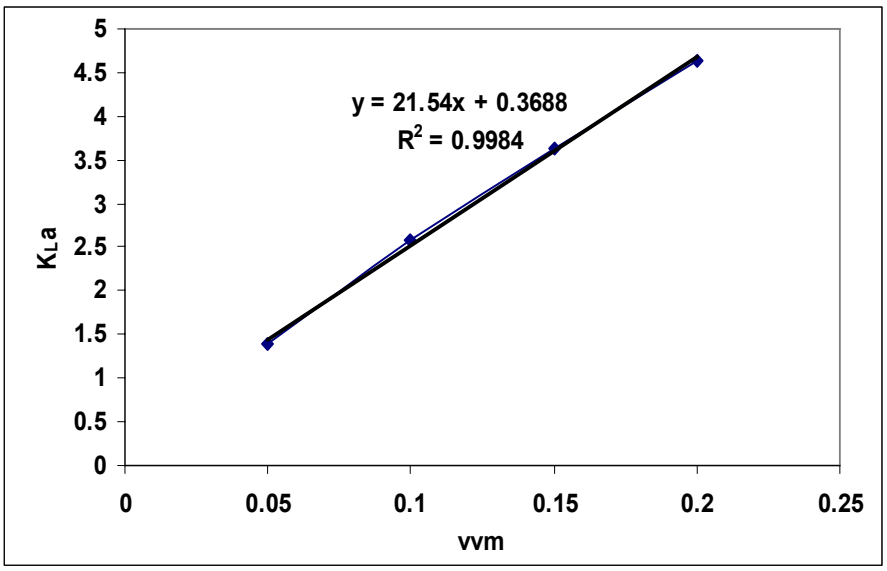


Figure 6.22 Correlation between oxygen transfer coefficient (K_La) at various aeration rates in the synthetic melanoidins-containing wastewater

The bacterial consortium could grow and showed decolorization activity around 18.92%, 19.27%, 16.94% and 8.31%, when cultivation at the aeration rate of 0 vvm (K_La=0.3688 h^{-1}), 0.1 vvm (K_La= 2.5836 h^{-1}), 0.2 vvm (K_La= 4.6343 h^{-1}), and 0.4 vvm (K_La= 8.9848 h^{-1}), respectively (Figure 6.23, 6.24, 6.25 and 6.26).

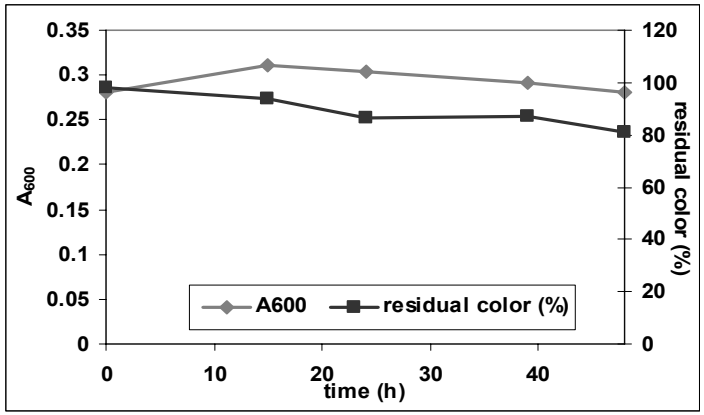


Figure 6.23 Time course of decolorization and bacterial growth using an aeration rate of 0 vvm (K_La=0.3688 h^{-1}).

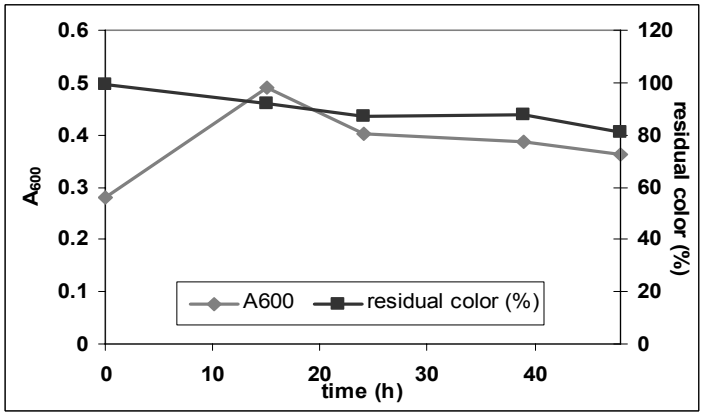


Figure 6.24 Time course of decolorization and bacterial growth using an aeration rate of 0.1 vvm (K_La= 2.5836 h^{-1}).

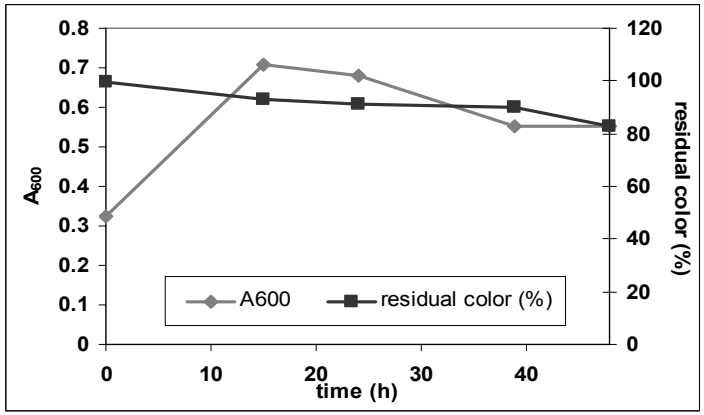


Figure 6.25 Time course of decolorization and bacterial growth using an aeration rate of 0.2 vvm (K_La= 4.6343 h^{-1}).

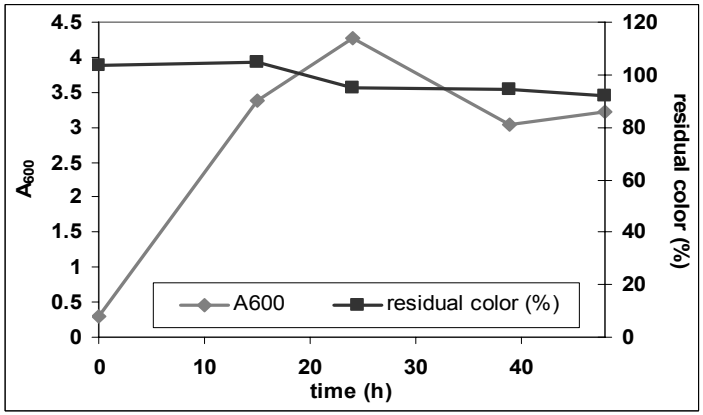


Figure 6.26 Time course of decolorization and bacterial growth using an aeration rate of 0.4 vvm (K_La= 8.9848 h^{-1}).

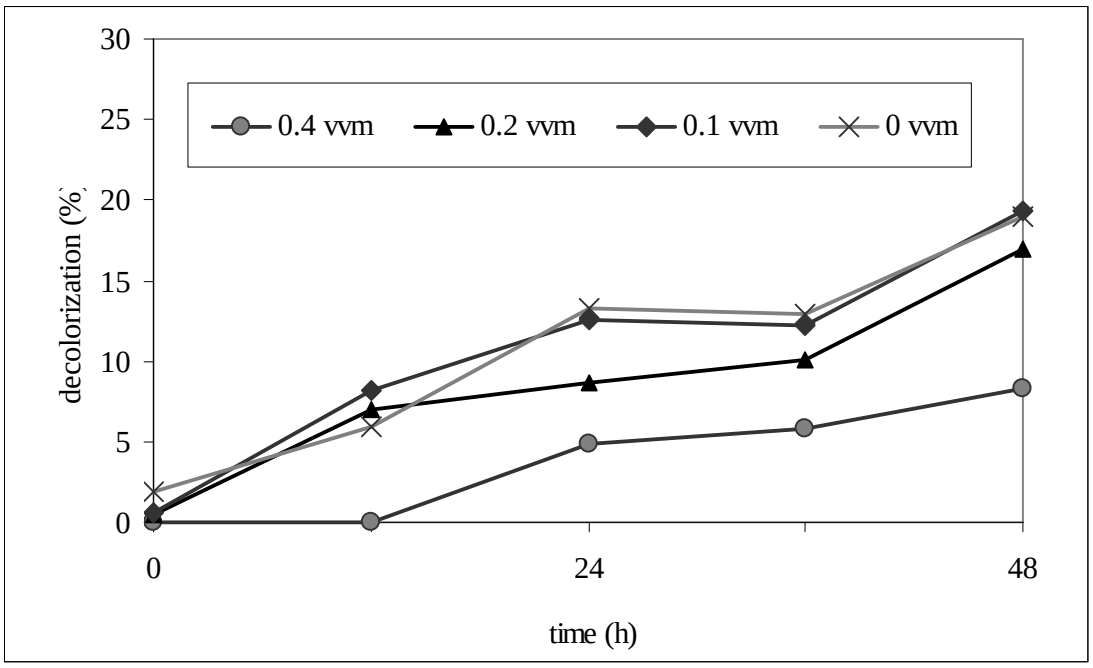


Figure 6.27 The effect of aeration rate on decolorization of synthetic melanoidins-containing wastewater containing 2% (v/v) Viandox as a color substance.

Figure 6.27 showed that the consortium could decolorize synthetic melanoidins-containing wastewater medium at aeration rate of K_La=0.3688 h^{-1} (0 vvm), K_La= 2.5836 h^{-1} (0.1 vvm), K_La= 4.6343 h^{-1} (0.2 vvm), and K_La= 8.9848 h^{-1} (0.4 vvm) up to 18.92%, 19.32%, 16.94%, and 8.31% within 48 h, respectively. Further increase in the aeration rate, instead of improving the decolorization, inhibited it. Finally, the best condition for decolorization seemed to be on closed to anaerobic condition. Then the aeration rate for further melanoidins decolorization was chosen as K_La= 2.5836 h^{-1} (0.1 vvm).

The explanation of this behavior may be found in the fact that the microorganisms that have been shown to degrade melanoidins are not exactly suitable for treating melanoidins-containing effluent from distilleries. However, the results presented in this study showed that color removal under low aeration conditions relatively higher than the highly aerobic condition. Hence, the decolorization mechanisms of melanoidins-containing wastewater by bacterial consortium MMP1 in this study might be due to metabolism of bacterial cell under facultative and anaerobic conditions such as fermentation and anaerobic respiration (Ames et al., 1999; Sirianuntapiboon and Prasertsong, 2008).

CHAPTER VII

COMPARATIVE STUDY FOR THE REMOVAL OF MELANOIDINS VIA BACTERIAL ADSORPTION AND DEGRADATION

Adsorption has been reported as an efficient method for the removal of different toxic pollutants and organic compounds in wastewater. Among various kinds of adsorbents, bacterial cells are widely used as powerful adsorbents for most pollutants in wastewater. It is also used as an adsorbent to remove color from the clarified juice in sugar refineries (Ruiz and Rolz 1971). These higher adsorption capacities were correlated to the surface nature of bacterial cells which are enriched in functional groups that act as adsorption sites for a variety of aqueous chemical species, including melanoidins (Beveridge 2005; Hass 2004). Because of their high adsorption capacities and low production costs, viable and dead bacterial biomasses have also drawn the attention of specialists in the field of water treatment (Sirianuntapiboon et al., 2004). Furthermore, only few studies have systematically compared the adsorptive properties of viable and dead bacterial cells for melanoidins-containing wastewaters.

Hence, this chapter describes the decolorization of the synthetic melanoidins-containing wastewater medium using viable and autoclaved cell of the constructed bacterial consortium MMP1.

7.1 Decolorization by living and autoclaved cells

This study was carried out to check whether the decolorization observed was due to biological or non-biological activity (abiotic decolorization). Living and autoclaved cells of bacterial consortium with different cells concentrations at 5–50% (v/v) were added into 250 ml Erlenmeyer flask, each containing 50 ml of synthetic melanoidins-containing wastewater medium. The flasks were placed on rotary shaker (200 rpm) at room temperature of 48 h. Samples were taken at regular time and then centrifuged at 10,000 rpm for 10 min. The supernatants were read at OD equal to 475 nm using a spectrophotometer. The summarized results of decolorization of the synthetic melanoidins-containing wastewater by living and autoclaved bacterial cells after incubation under aerobic conditions for 48 h are shown in Figure 7.1. It showed that the experiment with the different initial cells concentrations (5–50% v/v) of autoclaved cell from consortium MMP1, exhibited no melanoidins decolorization after incubation for 48 h. On the contrary, the melanoidins decolorization was observed with living cell of MMP1.

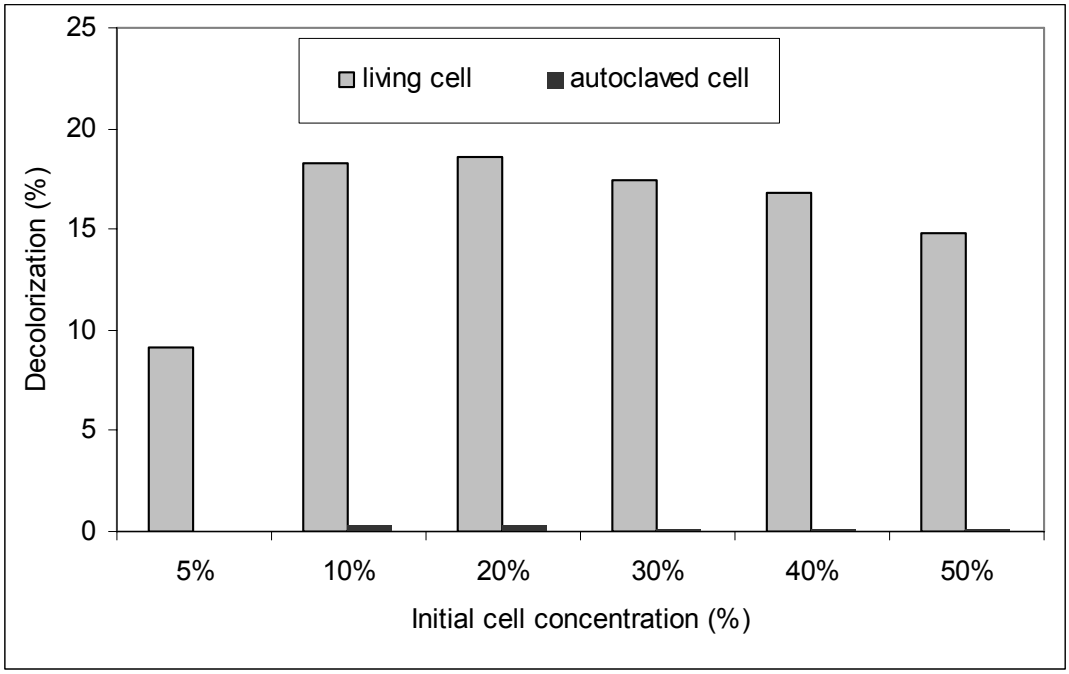


Figure 7.1 Decolorization assays of synthetic melanoidins-containg wastewater with different initial concentration using either viable cells or autoclaved cells of the constructed bacterial consortium MMP1. The data were averaged from three independent experiments.

The effect of different concentrations of inoculum on decolorization and growth of constructed bacterial consortium MMP1 are shown in Figures 7.2 – 7.3, respectively. It shows the percentage of decolorization corresponding to the initial concentration of bacterial cells. The results in Figure 7.3 indicate that the decolorization varied markedly with the initial bacterial concentration, in the range of 9-18% after aerobic incubation for 48 h. Individually, initial bacterial concentration at 5%, 10%, 20%, 30%, 40% and 50% could decolorize melanoidins-containing wastewater up to 9.1%, 18.2%, 18.5%, 17.4%, 16.8% and 14.8%, respectively after aerobic incubation for 48 h (Figure. 7.3). The results show that the initial cells concentration at 10% and 20% accounted for the majority of decolorization of synthetic melanoidins-containing wastewater. The lowest decolorization was observed when the initial 5% cells concentration was used.

As observed in Figures 7.4 - 7.5, the adsorption capacities of autoclaved cells from the constructed bacterial consortium MMP1 constituted of *Klebsiella oxytoc*a, *Serratia mercescens*, and the unknown bacterium DQ817737 for melanoidins were markedly lower compared to those of viable cells. The decolorization of autoclaved cells at all different initial cells concentrations was limited to 0.9% by adsorption of the bacterial cells (Figure 7.5).

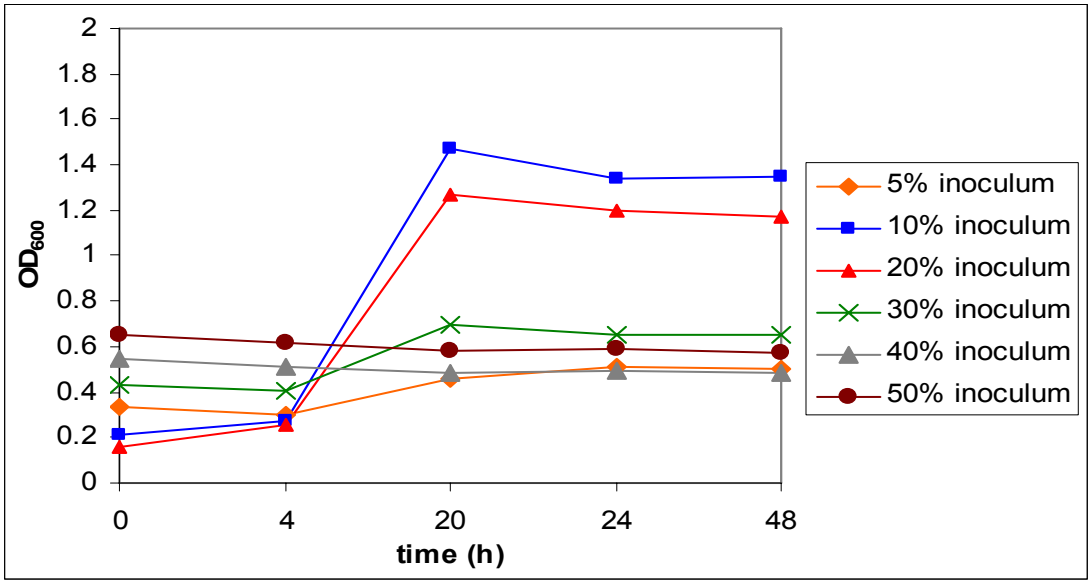


Figure 7.2 Effect of different initial concentrations of seed inoculum on the growth of the constructed bacterial consortium MMP1.

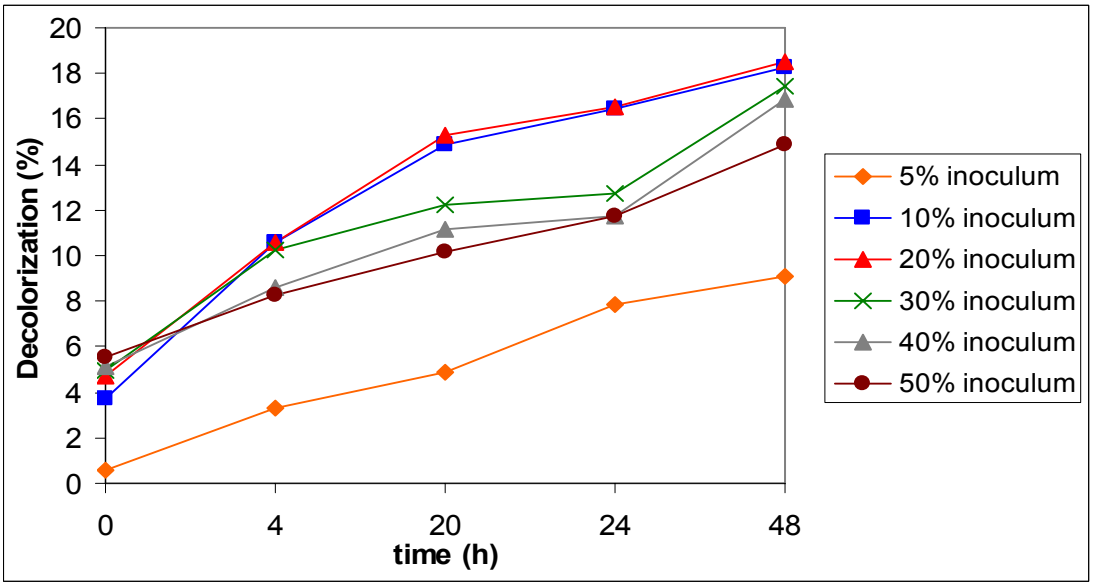


Figure 7.3 Effect of different initial concentrations of seed inoculum on the decolorization of the synthetic melanoidins-containing wastewater.

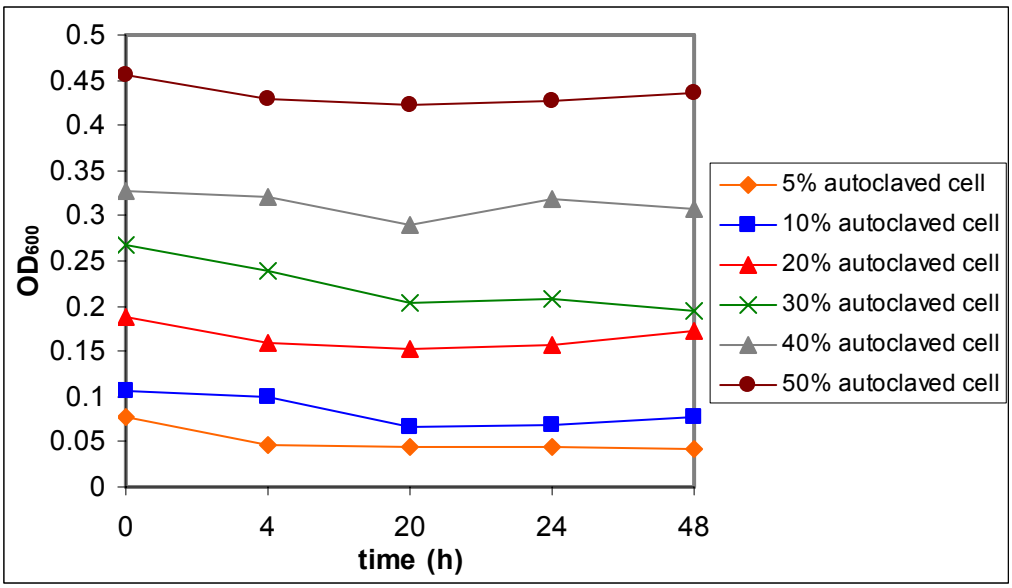


Figure 7.4 Effect of different initial concentrations of autoclaved cells on biomass of the constructed bacterial consortium MMP1.

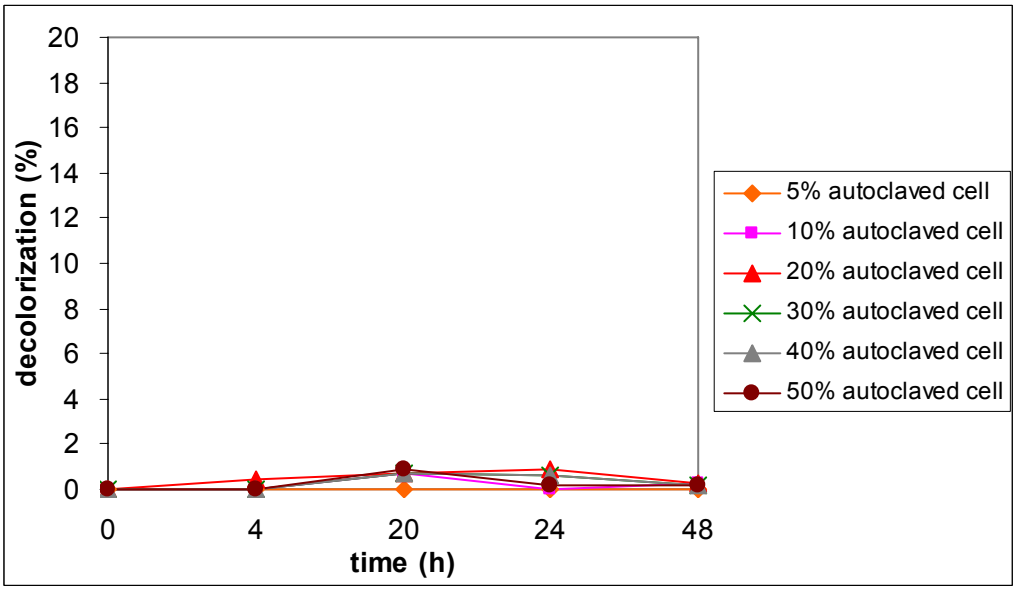


Figure 7.5 Effect of different initial concentrations of autoclaved cells on the decolorization of the synthetic melanoidins-containing wastewater.

7.2 Elution of adsorbed-melanoidins from bacterial cells

Additionally, to confirm that the melanoidins decolorization occurred by biological activity but not by adsorption mechanism, NaOH extraction was applied (Sirianuntapiboon et al., 2004). The cell pellets of either living cells or autoclaved cells sampled in a given volume, were resuspended with in the equal volume of NaOH 0.1 M to extract color substances adsorbed on cells surface. The extracts were centrifuged and measured at OD equal to 475 nm. The figure 7.6 showed that the final fractions of NaOH-extractable color substances were negligible. These results clearly indicated that the decolorization of melanoidins by consortium MMP1 was mainly due to biological mechanisms.

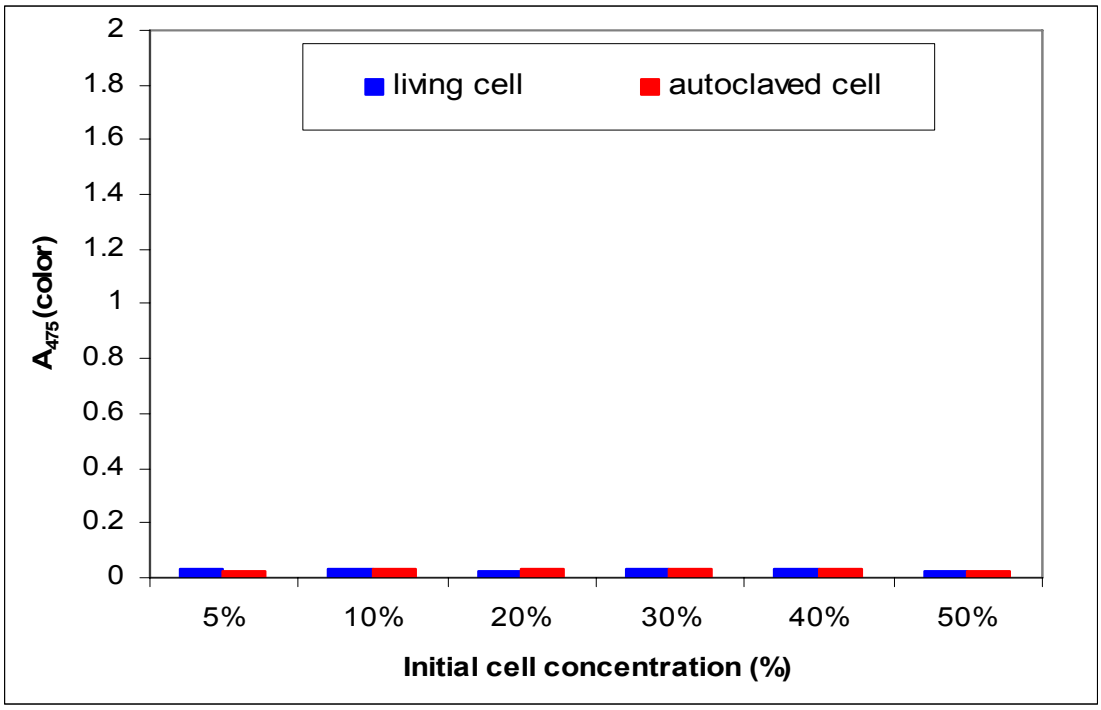


Figure 7.6 Release of melanoidins compounds from viable and autoclaved cells of constructed bacterial consortium MMP1 after extraction with 0.1M NaOH. The data were averaged from three independent experiments

The comparison of decolorization of consortium MMP1 with abiotic control has proved that the color removal for synthetic melanoidins-containing wastewater medium containing 2% (v/v) Viandox was due to the biotic activity of bacteria but not to the adsorption of color substances on the cells surface.

CHAPTER VIII

INVESTIGATION ON THE LIMITATION OF DECOLORIZATION EFFICIENCY

8.1 Investigation on the limitation of decolorization efficiency

It has been reported that melanoidins decolorization was dependent on bacterial growth conditions such as pH, nutrient levels, aeration and metabolites in liquid phase (Alkane et al., 2006; Ames et al., 1999; Gomaa et al., 2003; Kim and Shoda, 1999). Nutrients availability and metabolites accumulation might result in growth limitation and thereby decreased melanoidins decolorization. The aim of the experiments presented in this chapter 8 was, therefore, to conduct in-depth investigations in order to point out some of the key factors affecting the long-term performance of bacterial decolorization. Further, based on that the previous mentioned experiments, some strategies to enhance decolorization were also proposed.

To clarify the limitation of decolorization of the bacterial consortium MMP1, the used bacterial cells and the used medium were separately submitted to further study. The bacterial consortium was inoculated into synthetic melanoidins-containing wastewater medium and cultivated with shaking (200 rpm) at 30^{O}C for 48 h. Cells were harvested by centrifugation (10,000 rpm, 10 min, 4^{O}C) and washed three times successively with sterile normal saline solution in order to eliminate the residual culture medium. Washed bacterial cells were resuspended in the fresh culture medium of the same volume and cultivated under condition as described above. Meanwhile, the used culture medium was centrifuged again at 10,000 rpm for 10 min at 4^{O}C to completely remove the bacterial cells, then inoculated with fresh bacterial cells (10% w/v) and cultivated under the same condition as described above.

It was observed in Figure 8.1 that used cells suspension of the bacterial consortium MMP1 demonstrated much improved decolorization efficiency after cultivation with fresh medium. After cultivation for 72 h, the acclimatized bacterial consortium showed 15.4% decolorization of fresh synthetic melanoidins-containing wastewater medium, however, the fresh cells suspensions of bacterial consortium showed only 3.3% decolorization in used culture medium at the same incubation time (Figure 8.2)

Firstly, batch decolorization experiment confirmed the significant melanoidins decolorization capacity of the acclimatized bacterial cells in fresh medium. However, the decolorization efficiency of fresh bacterial consortium MMP1 in used medium was

significantly higher than that one observed in the experiment of used cells in fresh medium after the first 24 hours of incubation. As shown in Figure 8.2, it was observed that fresh cells suspension of the bacterial consortium MMP1 could decrease the color remaining in used culture medium after the first 24 hours of incubation. However, its decolorization efficiency dropped significantly after some hours of great decolorization. One of the possible causes of this phenomenon is might be due to the effect of toxicity of metabolites, which had been formed and accumulated during decolorization, thereby repressed the decolorization ability of fresh cells (Manjinder et al., 2005).

In general, the use of fresh cells suspension in used medium may not be an option for the continuous treatment of toxic compounds. Once the concentration of toxic compounds becomes too high or the process is operated for a long time, the amount of the original compounds and their metabolic products accumulated will reach saturation (Eccles, 1995). Beyond this point, the metabolism of living bacterial cells may be interrupted, resulting in death of the cells.

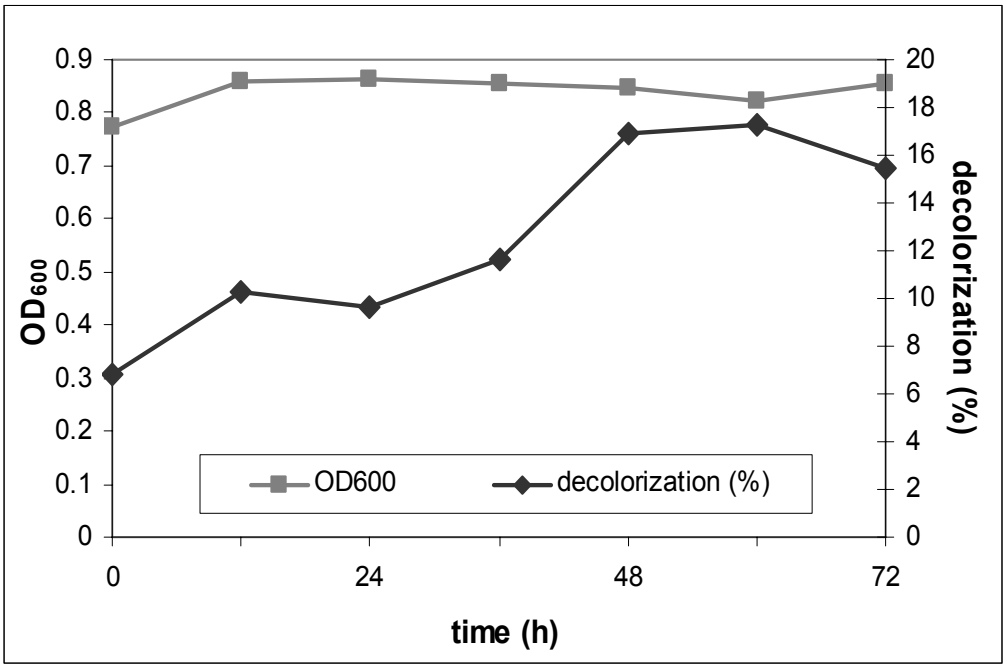


Figure 8.1 Limitation study of melanoidins decolorization by used bacterial cells in fresh synthetic melanoidins-containing wastewater medium.

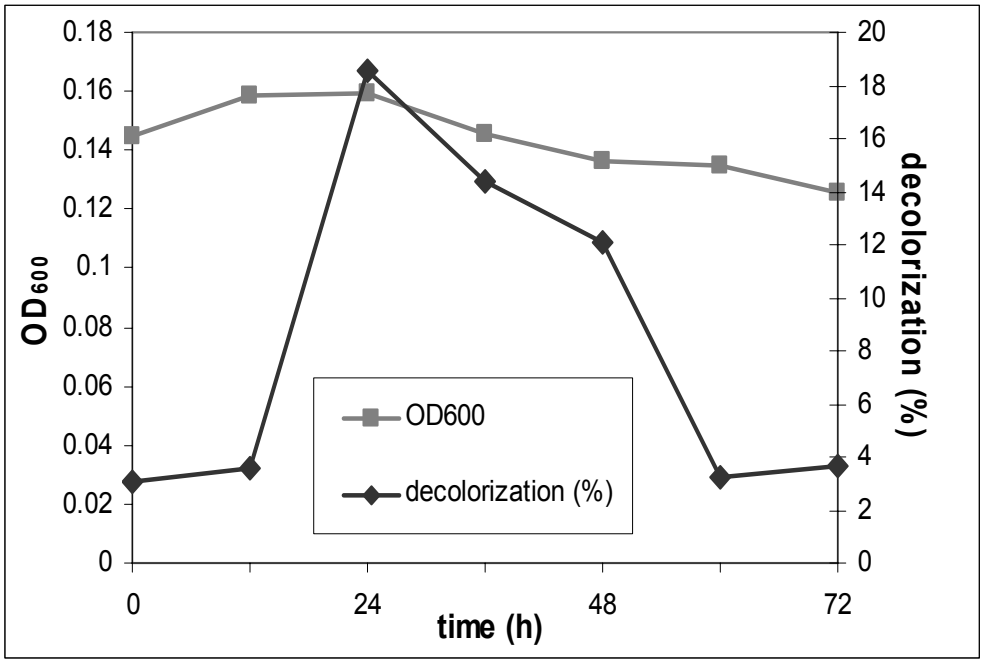


Figure 8.2 Limitation study of melanoidins decolorization by fresh bacterial cells in used synthetic melanoidins-containing wastewater medium

The result in Figure 8.2 indicated that the fresh cells suspension of bacterial consortium MMP1 could hardly removed the color in used synthetic melanoidins-containing wastewater medium during a given period. It was possible that the absence of nutrients markedly affected the decolorization of bacterial consortium MMP1. Adequate nutrients concentrations, including metal ions and vitamins, are required to support mixed bacterial cultures in wastewater treatment system in order to support viability of microbial community. In the cases where bacterial communities are dealing with a nutrient-limited wastewater, supplementation with nutrients can result in an enhanced degradation of pollutants (Singleton, 1994). Thus, in order to avoid decolorization efficiency drop, the effect of nutrient supplemented into used culture medium has been investigated in further study.

8.2 Nutrient Supplements for Optimization of the Bacterial Decolorization

Adding nutrients to biological treatment processes is one possible approach to upgrading an existing facility in order to deal with increasing volumes and strengths of industrial wastewaters. Usually, growth required macronutrients consisting of carbon, oxygen, hydrogen, nitrogen, phosphorus and sulfur (Valo et al.,

1985; Singleton, 1994) and micronutrients including vitamins and trace elements (Lind, 1994; Lemmer et al., 1998). Burgess et al. (1999) have reported that the addition of micronutrient have beneficial effect to support microbial growth in activated sludge to treat wastewater as an unbalanced activated sludge community can lead to sludge handling problems.

Various studies on melanoidins decolorization by microorganisms have shown the similar results regarding to the effect of nutrient supplements. Melanoidins decolorization of 87% was reported after 12 days of incubation with *Geotrichum candidum* in the presence of 2% glucose and inorganic nutrients (Kim and Shoda, 1999). Removal of melanoidins from molasses waste of 84.16% using *Aspergillus niger* in the presence of glucose has also been reported (Gomaa et al., 2003). Although higher decolorization could be achieved using additional nutrient supplement but this might lead to addition of extra chemicals in the system (Gomaa et al., 2003).

The present study extends the earlier work investigating the limitation of decolorization of the synthetic melanoidins-containing wastewater medium by the constructed bacterial consortium MMP1. Previous studies, have shown that the limitation of decolorization might be due to the lack of nutrients required for microbial growth and metabolic activity. In this study, it has been hypothesized that addition of macro- and micronutrient may overcome these problems, thus increasing the decolorization of bacterial consortium MMP1.

To confirm whether the limitation of the synthetic melanoidins-containing wastewater decolorization was from the lack of macro- and micronutrients or toxicity of metabolites, the experiment was carried out by addition of either 0.5% (w/v) LB or B vitamins into used synthetic melanoidins-containing wastewater medium. Eight B vitamins were chosen as follows: 0.2 mg/l of ρ-aminobenzoic acid, 0.4 mg/l of pyridoxine-HCl, 0.2 mg/l of thiamine-HCl, 0.2 mg/l of riboflavin, 0.2 mg/l of nicotinic acid, 0.2 mg/l of vitamin B_{12}, 0.08 mg/l of biotin and 0.08 mg/l of folic acid. It was observed that addition of 0.5% (w/v) LB (Figures 8.3) or B vitamins (Figures 8.4) could effect growth and melanoidins decolorization efficiency of bacterial consortium MMP1.

It can be seen from Figure 8.3 that the addition of 0.5% (w/v) LB increased the growth of bacterial consortium in reused culture medium. However, the result shows that the addition of 0.5% (w/v) LB into reused synthetic melanoidins-containing wastewater medium can not improve the decolorization efficiency of the bacterial consortium MMP1.

The addition of eight B vitamins into reused synthetic wastewater had inhibitory effects on the bacterial consortium as reflected in the color removal and growth rates (Figure 8.4). It was possible that the vitamin B markedly affected the decolorization of bacterial consortium MMP1. Also, it might be due to the effect of toxicity of metabolites, which had been formed and accumulated during decolorization, thereby repressed the decolorization ability of fresh cells (Manjinder et al., 2005).

As shown in Figure 8.3 and 8.4, the addition of 0.5% (w/v) LB and vitamin B provided the similar results and their decolorization did not increase significantly compared to the reused culture medium without nutrient supplementation. Therefore, the limitation of decolorization efficiency of the bacterial consortium MMP1 in this study was not directly due to a nutrient-l**imitation.**

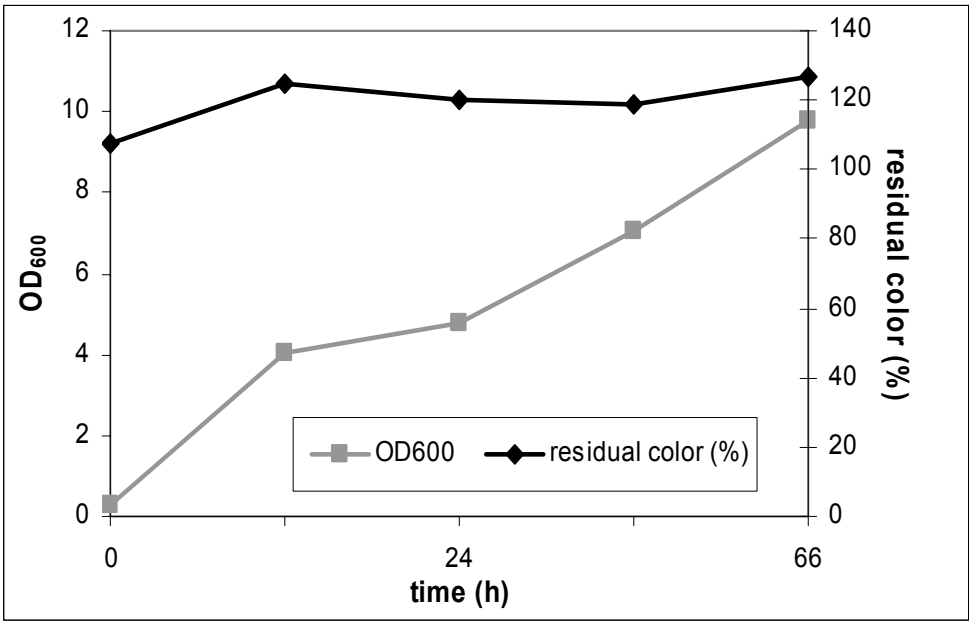


Figure 8.3 Melanoidins decolorization and growth of fresh bacterial cells in used synthetic melanoidins-containing wastewater medium supplemented with 0.5% (w/v) LB.

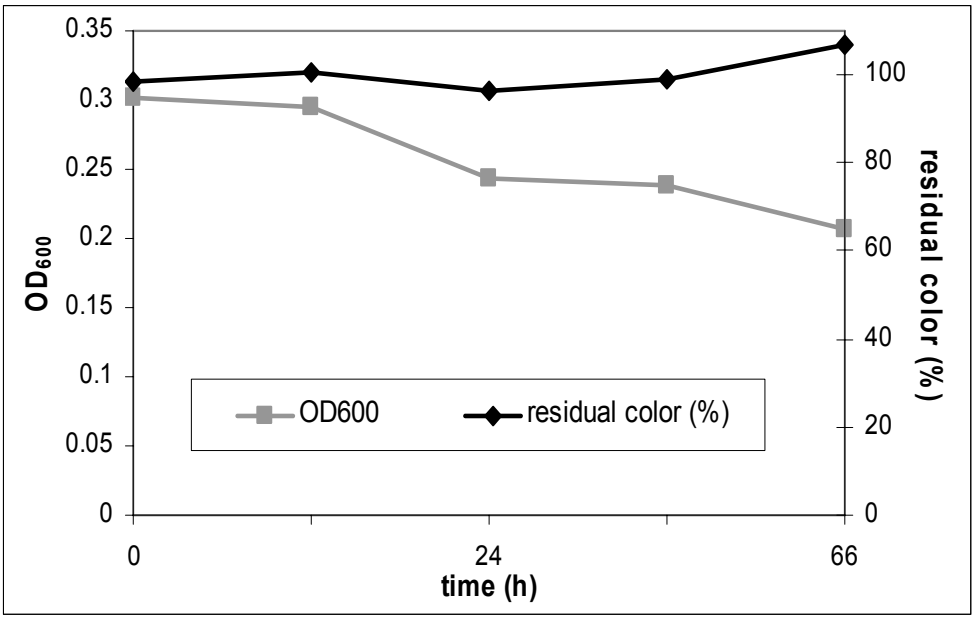


Figure 8.4 Melanoidins decolorization and growth of fresh bacterial cells in used synthetic melanoidins-containing wastewater medium supplemented with eight B vitamins.

8.3 Effects of trace elements on decolorization

One of the important differences between decolorization experiments which were carried out in different places, in Thailand and in France, may be the composition of trace elements in the tap water of each country. To prevent the effect of trace elements variation in synthetic melanoidins-containing wastewater medium and hence its suitability for bacterial decolorization, the decolorization experiments were carried out in 3 different synthetic melanoidins-containing wastewater media which were prepared by using different water sources as follow; double distilled water (DDW), tap water in Thailand and tap water in France, respectively.

The results in Figures 8.5 to 8.7 indicated that the bacterium consortium MMP1 could grow and showed decolorization in synthetic melanoidins-containing wastewater medium which was prepared with Tap water (in France) higher than the medium which was prepared with double distilled water (DDW). The decolorization obtained in the synthetic melanoidins-containing wastewater which were prepared by using double distilled water, tap water in Thailand, and tap water in France after incubation for 72 h were 16.4%, 14.5% and 27.5% (Figures 8.5, 8.6 and 8.7), respectively. The comparison of the decolorization of the bacterial consortium MMP1

in the synthetic melanoidins-containing wastewater medium which was prepared by tap water in France clearly revealed the presence of trace elements associated with bacterial decolorization.

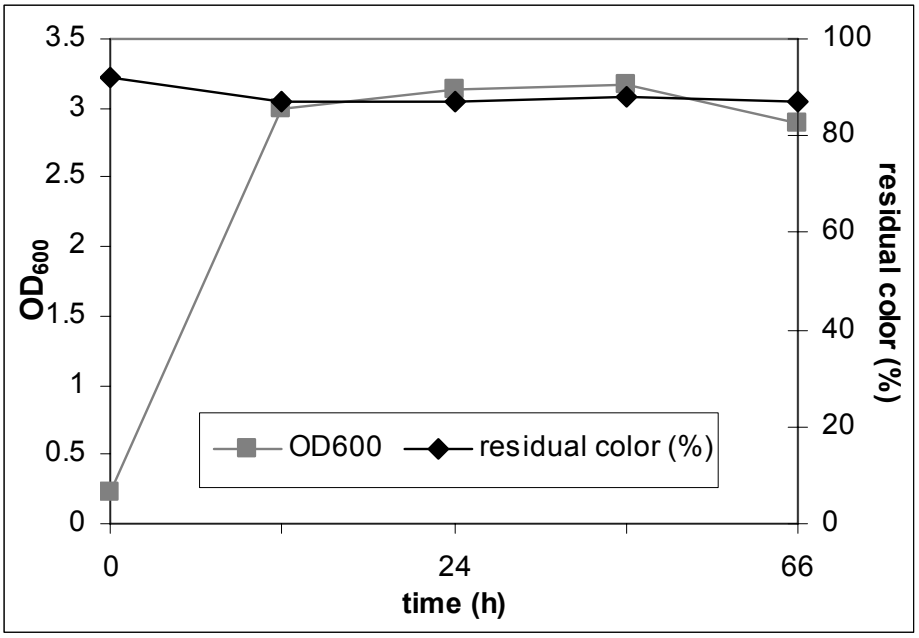


Figure 8.5 Growth and residual color of synthetic melanoidins-containing wastewater medium prepared with distilled water at different incubation times.

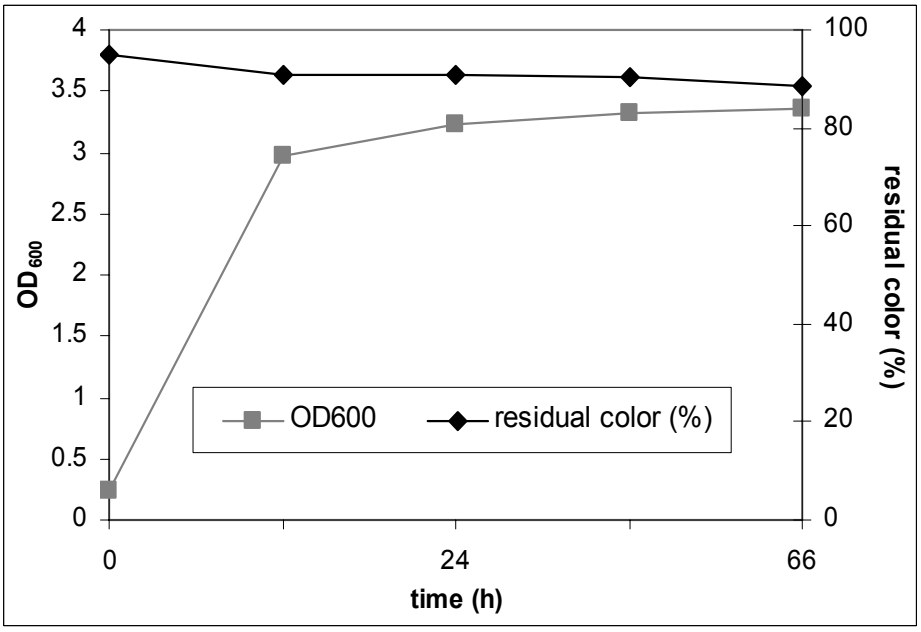


Figure 8.6 Growth and residual color of synthetic melanoidins-containing wastewater medium prepared with tap water in Thailand at different incubation times.

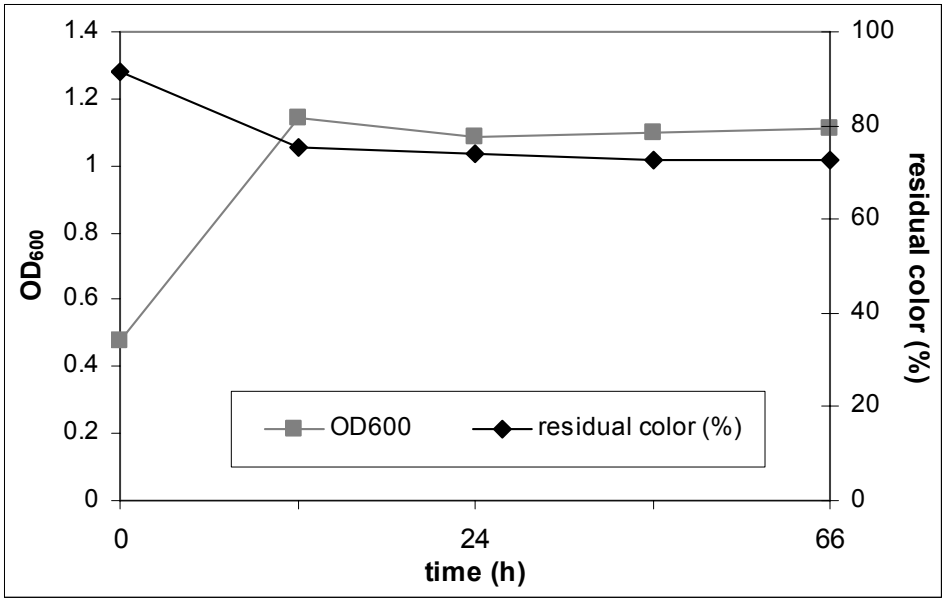


Figure 8.7 Growth and residual color of synthetic melanoidins-containing wastewater medium prepared with tap water in France at different incubation times.

Because of the higher decolorization, tap water in France had also drawn the attention of author to further experiments. Moreover, the different compositions of trace elements in tap water in Thailand and France might have variable effects on decolorization of bacterial consortium MMP1. Mahler and Cordes (1966) have reported that trace elements are taken up as components of enzymes and maintenance of enzyme structure. In the other hand, in some cases, excess trace elements have toxic effects (Madoni et al., 1996)

Hence, in order to elucidate the probable effect of trace elements on decolorization activity, the experiments were carried out. At the first step, the trace elements analysis of tap water in France was determined by ICP-Spectroscopy and the summaries of the roles of some trace elements indicated in Table 8.1. From ICP-spectroscopy results, the key trace elements of tap water in France were identified as follow; calcium, sulfur, sodium, magnesium, potassium at the concentration of 44.3, 31.0, 9.3, 4.3, and 1.3 mg/L, respectively. Then, the synthetic melanoidins-containing wastewater medium was prepared by using water which had key trace elements similar to tap water in France. To mimic the key trace elements available in tap water in France, the double distilled water were added with Na_2SO_4, $(NH_4)_2SO_4$, $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$ and KCl at the concentration of 28.85, 75.08, 162.27 and 44.12, 2.42 mg/L, respectively. The distilled water supplemented with trace elements was used as a water source for preparation of the synthetic melanoidins-containing wastewater medium hereinafter.

The growth and decolorization efficiency of bacterial consortium MMP1 in synthetic melanoidins-containing wastewater medium supplemented with trace elements was showed in Figure 8.8. The result indicated that the bacterial consortium showed lower growth and decolorization in synthetic melanoidins-containing wastewater medium prepared with double distilled water containing trace elements (Figure 8.8) compared to culture medium which was prepared by double distilled water (Figure 8.5). Trace amount of decolorization activity at 12.4% was also observed in the synthetic melanoidins-containing wastewater medium prepared with DDW containing trace elements after incubation for 72 h.

It appeared that the trace element supplementation did not improve decolorization efficiency of the bacterial consortium MMP1. Therefore, further decolorization experiments were carried out by using the synthetic melanoidins-containing wastewater medium prepared with distilled water without any supplementary trace element.

Table 8.1 Trace element requirements and the concentrations present in tap water in France

Trace elements	Reported requirements (mg/L)	Role of trace metal	Concentration detected (mg/L)
Ca	0.4-1.4	Cell transport systems and osmotic balance in all bacteria. Bridging anionic ECP and aiding flocculation. Increase growth rates. Requirements and effects vary.	44.301
K	0.8->3.0	Cell transport systems and osmotic balance in bacteria.	1.268
Fe	0.1-0.4	Growth factor in bacteria, fungi and algae. Adsorbed in proportion to the concentration available. Electron transport in cytochromes. Synthesis of catalase, peroxidase and aconitase.	0.004
Mg	0.4-5.0	Enzyme activator for a number of kinases and phosphotransferase in heterotrophic bacteria.	4.350
Mn	0.01-0.5	Activates bacterial enzymes. Often interchangeable with magnesium in kinase reactions. Lower affinity for binding sites than other metals but still can inhibit metabolism at 1 mg/L	0.002
Cu	0.01-0.5	Bacterial enzyme activator required in trace quantities. Can inhibit metabolism. Chelates other substances, reducing their toxicity.	0.059
Zn	0.1-0.5	Bacterial metallic enzyme activator of carbonic anhydrase and carboxypeptidase A. Dissociable on active site of enzymes. Stimulates cell growth. Can exacerbate toxic effects of other metals and inhibit metabolism.	0.491
Mo	0.2-0.5	Molybdenum is a common limiting nutrient	0.043
Co	0.1-5.0	Bacterial metallic enzyme activator. Dissociable on active site of enzymes. Activates carboxypeptidase for synthesis of vitamin B12 but otherwise toxic. Can inhibit metabolism.	0.001

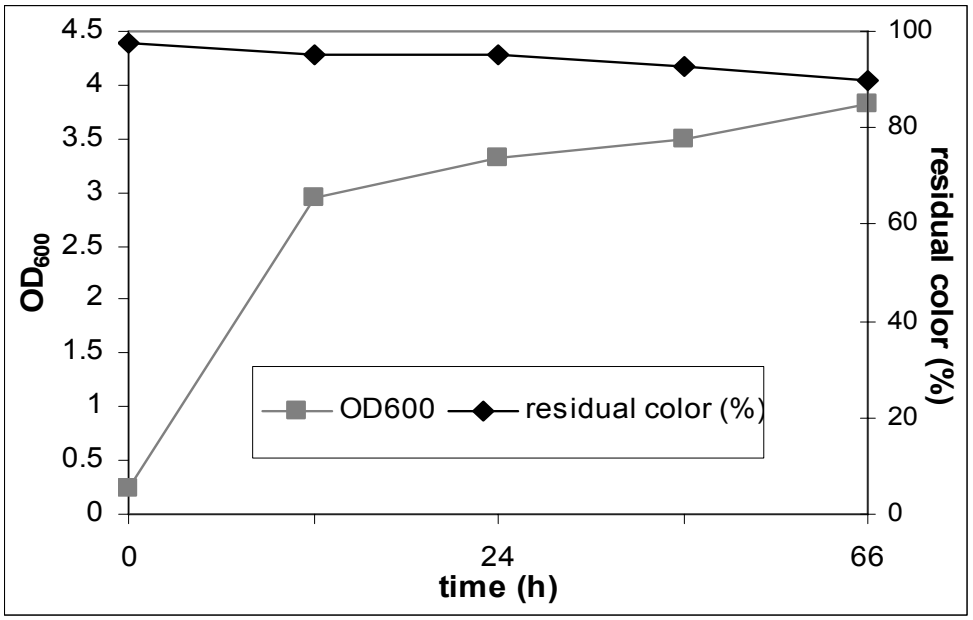


Figure 8.8 Growth and residual color of synthetic melanoidins-containing wastewater medium prepared by using DDW supplemented with trace elements at different incubation times.

In this chapter, the limitation of decolorization efficiency of bacterial consortium MMP1 was investigated. These results indicated that the nutrient supplement did not show significant effect on melanoidins decolorization of bacterial consortium MMP1, the addition of 0.5% (w/v) LB had supplementary effect so that the growth of bacterial consortium had a much higher than used culture medium without nutrient supplementation.

The effect of trace elements on decolorization of bacterial consortium MMP1 in various synthetic melanoidins-containing wastewater media has been investigated, and such results would be required when selecting water sources for the preparation of culture medium at different places in the world. The data indicated that the impact of understanding the nutrients required for melanoidin decolorization could be useful to optimize bacterial efficiency for the proposed treatment of melanoidins-containing wastewaters.

CHAPTER IX

DECOLORIZATION OF MELANOIDINS-CONTAINING WASTEWATER IN MEMBRANE BIOREACTOR

In recent years, membrane technology has emerged as a feasible alternative for convenient treatment of wastewater. Coupled with bacterial treatment processes, membrane technology has gained considerable attention due to its wide range of applicability and the performances of membrane systems that have been established by various investigations during the last decade.

Membrane bioreactors (MBRs), the combination of membrane separation with a suspended growth bioreactor, is a convenient technology for wastewater treatment since it posses advantages such as complete removal of the suspended solids and macro-colloidal material, prolonged microbial retention time (sludge retention time, SRT), reduction in costs, easiness for control of reactor operating conditions. According to these positive aspects, MBR has been applied to various wastewater treatments to achieve higher effluent quality, which is often difficult to be effectively met by conventional activated sludge process. (Judd, 2006; Stephenson et al., 2000; Yun et al., 2006).

In this way, the advantages of MBR are a high mixed liquid suspended solids (MLSS) concentration, a lower excess sludge production. Moreover, the produced treated water can be reused (Meng et al., 2008). High solid retention times (SRTs) enable one to increase the sludge concentration and the applied organic load, thereby increasing the pollutant degradation. This allows the development of microorganisms able to remove highly recalcitrant pollutants contained in wastewater, resulting in improved removal rate.

The main aim of membrane bioreactors is to improve the efficiency of the biological process step for obtaining a high-quality effluent. Because biological treatment and membrane separation are rather distinct processes, the combined MBR process is relatively complex. To optimize the MBR process, many parameters have to be considered. These include the aeration rate and hydraulic retention time (HRT) in the biological step as well as the flux rate, and the membrane materials in the membrane separation performances.

The combination of bacterial decolorization and membrane technology should represent a more promising technology for melanoidins-containing wastewater

treatment. Hence, in this chapter, a feasible system may be envisaged by coupling the decolorization capability of bacterial consortium with inherent advantages of membrane bioreactor. The constructed consortium MMP1 was selected as an inoculum for this study. The decolorization performances of the membrane bioreactor are studied to determine the optimum operating conditions, which will be applied to continuous decolorization of a synthetic melanoidins-containing wastewater.

In this study, the effect of operating parameters on melanoidins-containing wastewater decolorization and on the performance of the MBR process for treating the molasses wastewater by bacterial consortium were investigated.

9.1 Investigation of decolorization by bacterial consortium using side-stream membrane bioreactor

In side-stream configuration, membrane filtration occurs outside the bioreactor through recirculation. By pumping the mixed liquor from the bioreactor at a high pressure into the membrane unit, the permeate passes through the membrane and the concentrate is returned to the bioreactor. Solid retention time (SRT) in MBR can be controlled completely independently from hydraulic retention time (HRT). Therefore, a very long SRT can be maintained resulting in the complete retention of useful bacteria and this results in greater flexibility of operation. In addition, a side-stream operation is evaluated as a way to provide the increased high surface shear that would enable the operation at high levels of permeate flux.

The membranes used by MBR have pore sizes such that water and most solute species pass through the membrane whilst other larger species, such as solids and microorganisms, are retained. Thus, selection of the membrane materials (polymer or ceramic) and module types (tubular or hollow fiber) plays an important role on the membrane flux achieved. With these aims of investigation, several investigation with polymeric (polysulfone) and mineral (zirconia) membranes have been tested for bacterial decolorization performed in MBR. These experiments were carried out in two runs. The first run was carried out with polysulfone hollow-fiber membrane bioreactor. For the second run, the decolorization was carried out in MBR equipped with the external mineral tubular membrane module.

The objective of this study was to determine the operating parameters that affected the melanoidins decolorization of the constructed bacterial consortium MMP1 in the side-stream laboratory-scale membrane bioreactor, in order to improve the decolorization rate. The experiments presented in this study were carried out in a

side-stream cross-flow membrane bioreactor for the aerobic treatment of a synthetic wastewater containing Viandox sauce 2% (v/v) as color substrates.

9.2 Decolorization of melanoidins-cont aining w astewater in poly sulfone hollow-fiber membrane bioreactor

In this study, the experiment was performed using a laboratory-scale side-stream membrane bioreactor represented in Figure 9.1. A 2L laboratory scale side-stream MBR with 1.6L of working volume was used to conduct this study. The reactor was connected to an external membrane module, containing a single tubular membrane. In this study, membrane module was a hollow-fiber membrane module (Polymem, Fourquevaux, France), whose main characteristics were listed in Table 9.1. The membrane was made of polysulfone with a pore size of 0.1 μm, and internal/external diameter of 0.4/0.7 mm. The effective membrane filtration area was 0.1 m^2.

The aeration was maintained at 0.1 vvm with agitation speed at 150 rpm. The membrane bioreactor was initially inoculated with the constructed bacterial consortium MMP1. Reactor mixed liquor was circulated through the membrane module by mean of a peristaltic pump (Masterflex I/P). A synthetic melanoidins-containing wastewater was then continuously introduced. A water level was used to control the influent pump and to keep the water level in the bioreactor constant. The control of temperature at $30^{O}C$ was obtained using thermostatic recirculation vessel. Samples were taken from the bioreactor and from permeate at regular time and then centrifuged at 10,000 rpm for 10 min. The samples were collected and stored in a refrigerator at 4 ^{O}C until analyses.

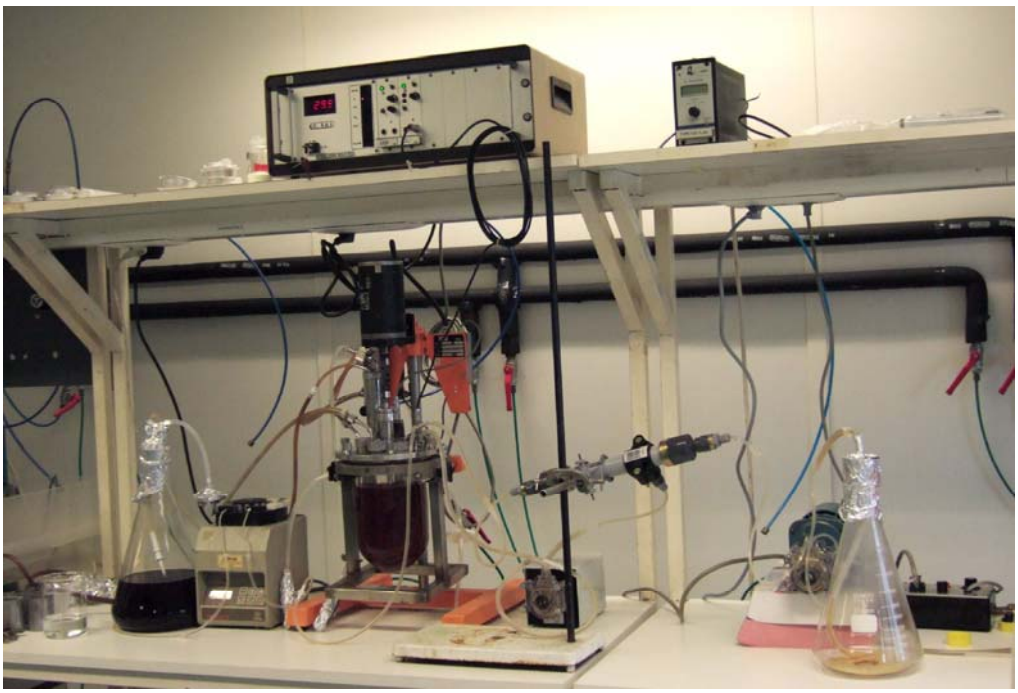


Figure 9.1 Decolorization experiment using polysulfone hollow-fiber MBR.

Table 9.1 Characterization of the polysulfone membrane (Polymem, France)

Classification	Characteristics
Membrane material	Polysulfone
Module type	Hollow-fiber
Internal/external diameter	0.4 mm /0.7 mm
Effective membrane filtration area	0.1 m^2
Mean pore size	0.1 μm

9.2.1 Decolorization experiment using polysulfone hollow-fiber MBR at HRT of 40 hours.

This experiment was performed in side-stream membrane bioreactor equipped with hollow-fiber membrane module by using HRT of 40 hours. Unfortunately, this experiment was stopped after 72 hours of operation because the membrane bioreactor was contaminated with fungi (Figure 9.2). Indeed, a major limitation of polysulfone hollow-fiber membrane bioreactor was the significant reduction of permeate flux significantly caused by membrane fouling. Membrane fouling can result from the precipitation of less soluble inorganic species, adsorption of organic substances (organic fouling), and adhesion and growth of microbial cells at the membrane surface (bio fouling). In this experiment, studies with polysulfone membrane showed contamination by biomass formed inside the reactor as shown in Figure 9.3. The explanation for the fouling of polysulfone membrane at very short operation time relied on the adhesion and growth of contaminated-microbial cells on the membrane surface, thus causing a major hydraulic resistance. This was a major problem in the polymeric membrane bioreactor because it reduced productivity, shortened membrane life and impaired the fractionation capability of the membrane.

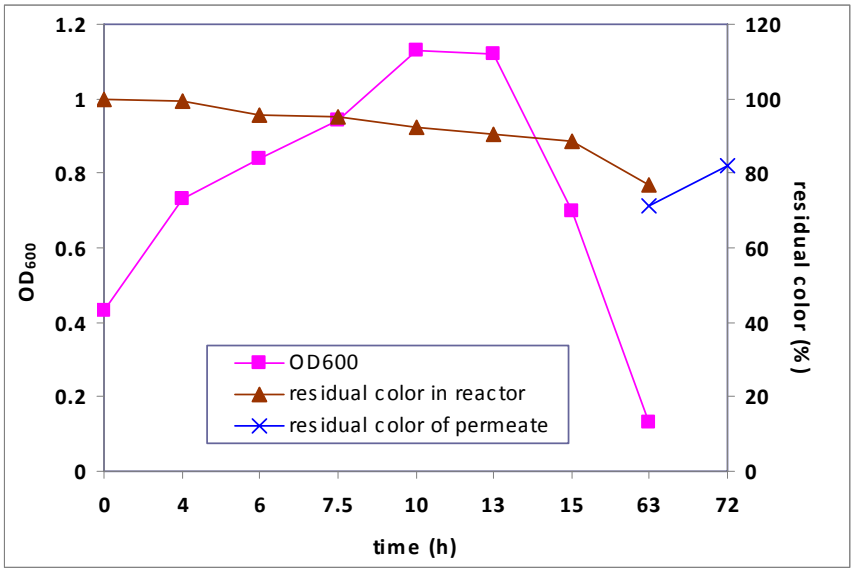


Figure 9.2 Time course of decolorization of the synthetic melanoidins-containing wastewater medium by the bacterial consortium MMP1 in the polysulfone hollow-fiber membrane bioreactor using a HRT of 40 h.

(a) (b)

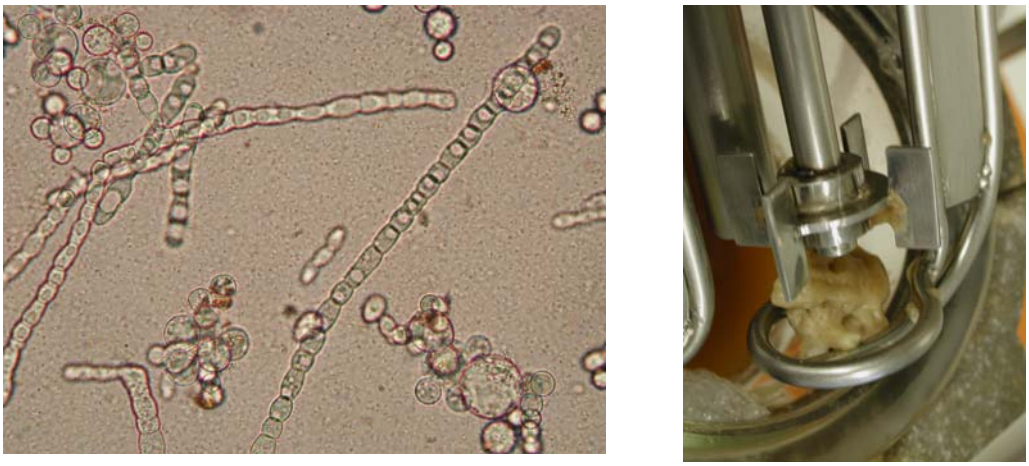


Figure 9.3 Microscopic photographs of contaminated microorganisms in MBR (a); and cake layer observed on the surface of the mechanical stirrer (b).

9.3. Decolorization of melanoidins-containing wastewater in mineral membrane bioreactor

Microfiltration mineral membrane bioreactor seems to be one of the most appropriate reactor to treat distillery wastewaters due to its permeation flux higher than polymeric membrane one. In addition, it is a more biologically compatible membrane material (Doyen et al., 1996). Moreover, it is not affected by solvents and also supports irradiation. These properties allow the easy cleaning of membrane by different processes. Continuous operation of this type of reactor is feasible as long as substrate solutions are supplied to the reactor. The advantages of mineral membrane bioreactors mentioned above make them suitable for operations of continuous processes. The aim of this part of the study is to quantify the performance of a mineral membrane bioreactor for treatment of melanoidins-containing wastewaters.

To investigate the decolorization performance of the bacterial consortium in mineral membrane bioreactor, the experiment was performed in an external membrane bioreactor. The 2L biological reactor contained 1.6L of synthetic melanoidins-containing wastewater was equipped with an aeration system composed of bottom-installed air nozzles. The decolorization experimental system of this study is represented in Figure 9.4 and flow diagram is showed in Figure 9.5. The membrane module was a stainless-steel monochannel (monotubular) microfiltration module, a mineral M14 Carbosep® membrane (Orelis, Miribel, France), whose main characteristics are listed in Table 9.2. The M14 membrane is a composite membrane

with a 0.14 µm mean pore diameter of zirconium dioxide (ZrO_2) and titanium dioxide (TiO_2) filtering layer on a carbon support (6 mm inner diameter, 60 cm long). This carbon-based zirconium membrane is highly resistant to pressure (up to 40 bars), to temperature (up to 350 ^{O}C), and pH (0-14).

There have been a number of investigations focused on the selection of suitable SRTs for wastewater treatment by MBRs. These reported results suggest that the optimum SRT of MBRs should be controlled at 20-50 days depending on HRT and feed wastewater (Han et al., 2005; Lee et al., 2003; Ng et al., 2006; Zhang et al., 2006). Thus, In this study, SRT at 50 days was selected for this study. The SRT was determined for 50 days by discharging calculated volume of sludge daily, in order to renew the reaction volume in 50 days.

The objective of this study was to determine the effect of different HRT on decolorization performance of the bacterial consortium MMP1. Three independent experiments were carried out at HRT of 40 hours, 20 hours, and 15 hours, respectively

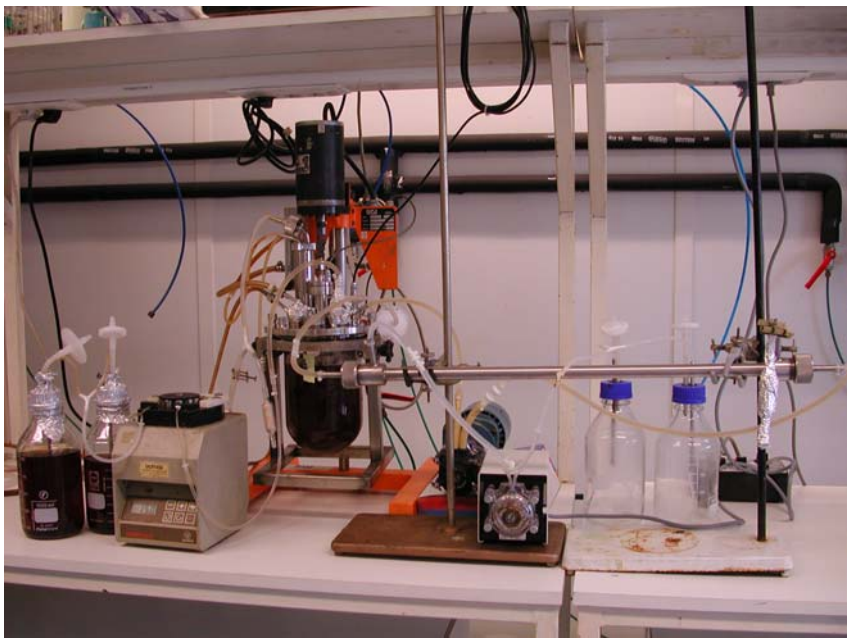


Figure 9.4 Decolorization experimental device : the external mineral MBR

Table 9.2 Characterization of the mineral membrane **(**Carbosep® membrane)

Classification	**Characteristics**
Mineral support	Carbon
Active layer	ZrO_2-TiO_2
Module type	Tubular
Length (m)	0.6
Internal/external diameter (mm)	6/10
Mean pore size (µm)	0.14
Effective membrane filtration area (m^2)	0.011
TMP (kPa)	<1,500

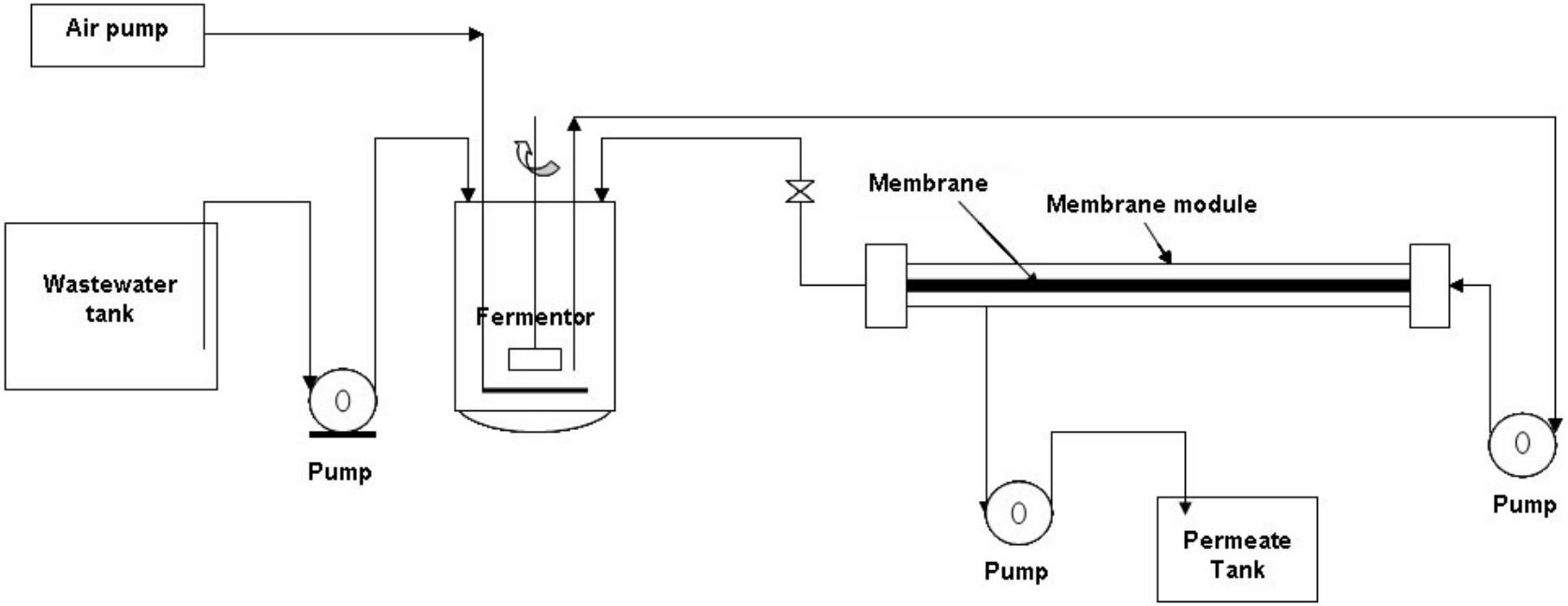


Figure 9.5 Flow diagram of decolorization of melanoidins system

In this study, all flows were sampled and analyzed weekly with the exception of the MBR effluent, which was sampled and analyzed twice weekly (i.e. every two or three days). Table 9.3 is the matrix that shows the organization of sampling frequencies and analyses for each flow.

Table 9.3 Testing matrix for laboratory testing

Material flow	Sampling	Parameters			
		OD_{475}	OD_{600}	DW	COD
Wastewater (Influent)		x	x		x
Bioreactor sludge	thrice/week	x	x	x	x
Permeate (Effluent)	thrice/week	x			x

9.3.1 Decolorization experiment using mineral MBR at HRT of 40 hours

Color variation with time

The MBR performance was initially investigated with HRT of 40 hours (Figure 9.6- 9.8). Figure 9.6 showed the evolution of biomass and color concentrations during the 50 days of operation. The absorbencies were obtained at 475 nm. The variation of permeate and color in reactor with time are shown in Figure 9.7. The results showed that the residual color of an effluent was consistently lower than the sludge supernatant color inside membrane bioreactor (Figure 9.7 and Figure 9.8). The color in the supernatant is increasing as if some accumulation would happen. Nevertheless, the color of the permeate is slightly lower than the initial one, and rather lower than the supernatant. This observation indicates an ability of the membrane to retain the colorizing molecules. This phenomenon happened after 10 days of operation, probably with an increase of the membrane fouling. Maximum decolorization of 53.53 % was observed in 2 days and the color rather increased until 8 days (185 h). Then, the bacterial consortium in the system could decolorize melanoidins again and later reached steady decolorization but without significant color reduction, because of the accumulation phenomena. After operation for 29 days, the decolorizing activity of bacterial consortium was constant approximately 33% and then the color was slowly increased until 50 days of operation. However,

the residual color in bioreactor was dramatically increased from 99.56% after 23 days of operation to 186.14% at the end of experiment (50 days).

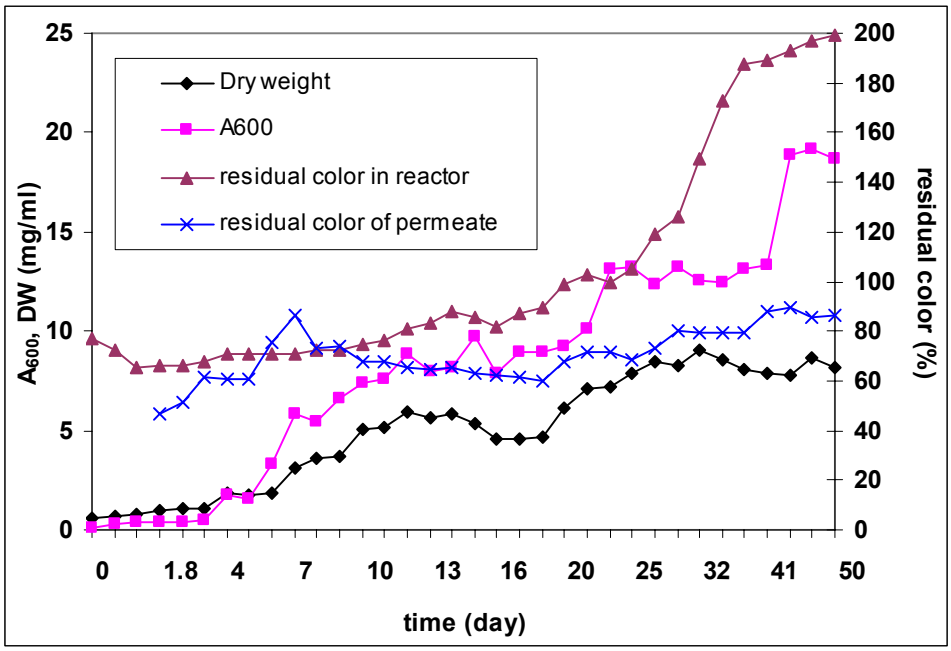


Figure 9.6 Time course of decolorization of the synthetic melanoidins-containing wastewater medium by the bacterial consortium MMP1 in mineral membrane bioreactor using HRT of 40 hours. The consortium was cultured under the conditions described in the text. Symbols: (▲), residual color of culture medium in bioreactor, (**x**) residual color of culture medium in permeate, (♦) bacterial dry weight and, (■) optical density at 600 nm.

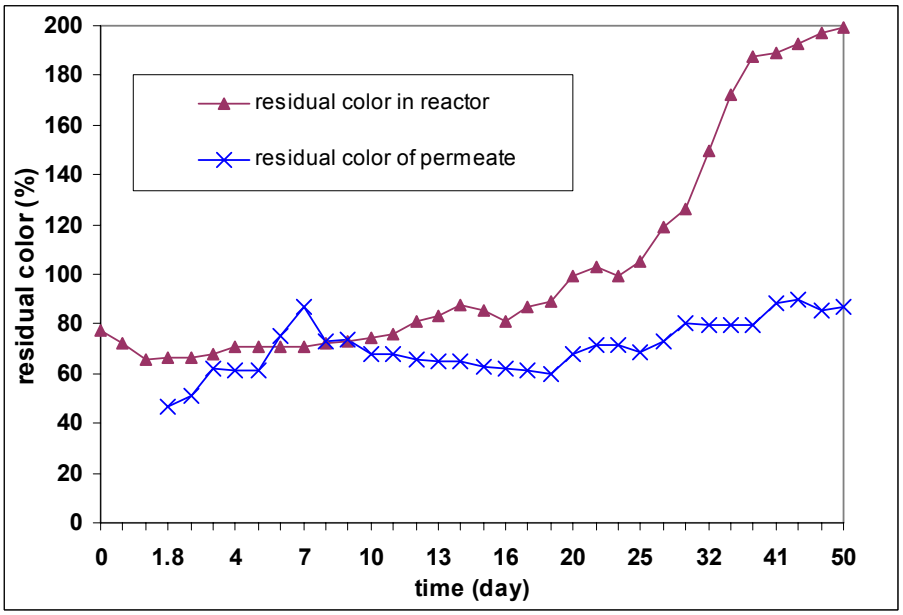


Figure 9.7 Variations of permeate and feed color with time in mineral membrane bioreactor which using a HRT of 40 hours.

Figure 9.8 Characteristics of the synthetic melanoidins-containing wastewater before treatment (left-hand) and the permeate of the mineral MBR (right-hand).

An increase in color intensity of wastewater within bioreactor might be due to the accumulation of colored compounds and/or the formation of new colored substances. It is possible that cell debris and some intracellular biomolecules such as proteins, carbohydrates, organic nitrogen, amino acids react with the residual colored compounds resulting in the formation of large molecular weight color compounds (Martins et al., 2000). The low COD removal result as shown in Figure 9.10 also indicates that the recalcitrant compound present in wastewater were not effectively degraded and possibly their concentration increase inside the bioreactors.

Melanoidins, the brown polymers formed through Maillard reaction, are most commonly present in molasses wastewater. The chemical properties of melanoidin are humic substances-like, being acidic, polymeric and highly dispersed colloids, negatively charged due to the dissociation of carboxylic and phenolic groups (Migo et al., 1993). In general, melanoidins are colloidal compounds of molecular weight between 40 and 70 kDa. Increasing degree of polymerization of melanoidins is reported to increase size of the colloids (Pena et al., 2003; Coca et al., 2005). In this study, the formation of colloidal particles had been observed in wastewater within bioreactor after 7 days of operation (Figure 9.9). Based on the above information,

thus, another possibility of color increasing in wastewater might be related to the formation of colloidal compounds within bioreactor.

Evidence supporting this idea is the difference of color intensity at 475 nm between wastewater sample in bioreactors and the permeate collected from microfiltration membrane modules with a pore size of 0.14 μm.

Figure 9.9 Characteristics of wastewater in bioreactor after 7 days of operation using HRT of 40 hours.

One more possibility in the higher color intensity (A_{475}) and turbidity (A_{600}) of supernatant in bioreactor after 20-days operation can be related to supernatant colloidal COD (Vogelaar et al., 2002). The increase in colloidal COD in bioreactor might be due to the increasing of free bacterial cells, cell components or extracellular polymeric substances (EPS). In this study, the concentration of COD in the supernatant of wastewater in bioreactor after 20-days operation was slightly higher than in the permeate (Figure 9.10). This indicates that the compounds that are retained in the bioreactor are colloidal COD and it is possible that their concentration increase in the long operating times.

These results showed that the color concentration of treated water required an optimization of the membrane bioreactor to improve the decolorization rate. It

must be noticed also that the microorganisms usually required an acclimatizing to the MBR process, lasting 2 or 3 times the SRT, which has not be respected here for time consuming reasons.

Sludge production

The growth of bacterial consortium MMP1 during the experiment is shown in Figure 9.6. Time course of effluent decolorization was studied along with the growth of the consortium MMP1. With the increase of time, there was an increase in cells mass but not decolorization. An increasing trend of bacterial biomass with time resulted mainly from the prolongation of SRT (compared to conventional process) and the retention by the membrane. The large fluctuations of biomass concentrations observed during 50-days operation could be attributed to bacterial acclimation in response to the changes in physico-chemical environments in this MBR (e.g. high shear stress from the recirculating pump). After each decline, the biomass was stabilized; this might be due to the adaptation of microorganisms in membrane reactor.

COD removal efficiency

The variation of supernatant inside reactor and effluent COD concentration with time during the experiment is illustrated in Figure 9.10. The COD of permeate and supernatant in reactor after 50 operation days remained at about 16,382 mg/L and 19,007 mg/L, respectively, although the influent COD was around 22,000 mg/L. Thus, the average removal of COD was 25.5%. In this study, though the biomass growth is satisfactory, COD reduction appears to be limited by the presence of recalcitrants (comprising the coloring compounds melanoidins) in the feed, which are known to restrict bacterial growth.

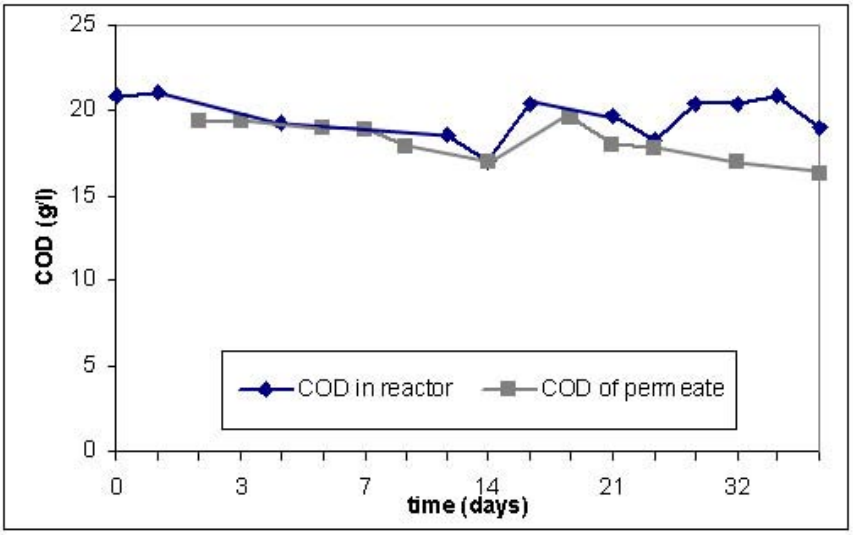


Figure 9.10 Variations of COD in bioreactor and permeate with time in mineral membrane bioreactor using HRT of 40 hours.

Membrane permeability

Permeate flux is influenced by a number of factors including the feed water (composition), the membrane (element geometry/configuration), area and material composition), and operation (hydrodynamics). It is critically determined by the tendency of the membrane to be fouled by feed water components owing to their accumulation on the internal and external structures of the membrane (Stephenson et al., 2000). Figure 9.11 shows the variation of permeability of the mineral membrane before and after operation with HRT of 40 h.

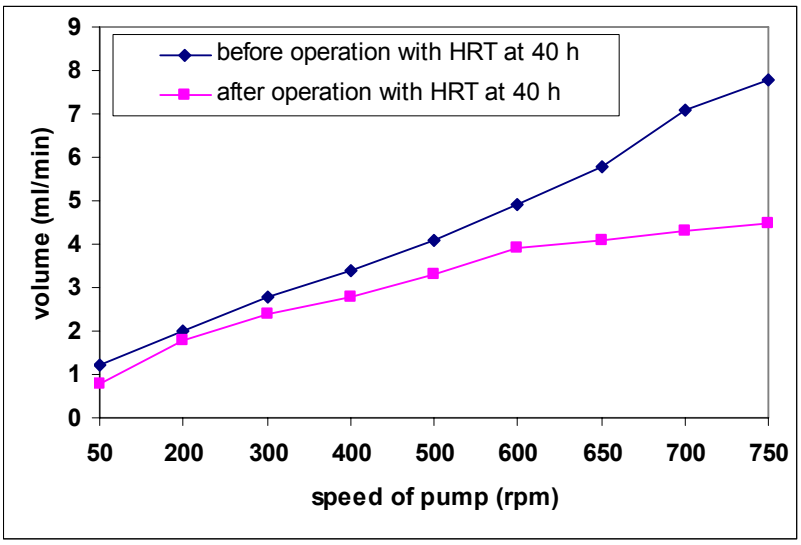


Figure 9.11 Variation of permeability (at 25^{O}C) of the mineral membrane before and after 50 days of operation with HRT of 40 hours.

The result of the water permeability test in Figure 9.11 shows that the permeability of mineral membrane was slightly decreased after 50 days continuous operation by using HRT of 40 hours. In general, the reduction of membrane permeability could be related to the increase of biomass concentration (Li *et al.,* 1984), the size reduction of mixed liquor biosolids (Bailey *et el.,* 1994), or the size distribution of particles being filtered (Chang *et al.,* 1994).

In this study, the decrease of membrane permeability after operation was probably due to the increase of bacterial biomass within the system and/or the formation of bacterial biofilm on the membrane surface which had been removed easily by washing or increasing fluid velocity.

9.3.2 Abiotic control of decolorization experiment using mineral MBR at HRT of 40 hours

Abiotic decolorization study was carried out to check whether the decolorization was obtained from biological activity or non-biological activity. The synthetic melanoidin wastewater was used as a control to analyze the performance of mineral membrane bioreactor.

Color variation with time

The variation of permeate and color in reactor with time is shown in Figure 9.12. The results showed that residual color of supernatant inside reactor was consistent. The residual color of effluent at 48 h was around 32 %, then, the color rather increased to 100% until the end of operation. The lower residual color of permeate in first 4 days of operation might result from the water used for washing and sterilization process remained inside the membrane and dilute the color of permeate. The comparison of decolorization of consortium MMP1 from previously study with abiotic control has proved that the color removal for synthetic melanoidins-containing wastewater medium containing 2% (v/v) Viandox was due to biotic activity of bacteria but not adsorption of color substances on the membrane.

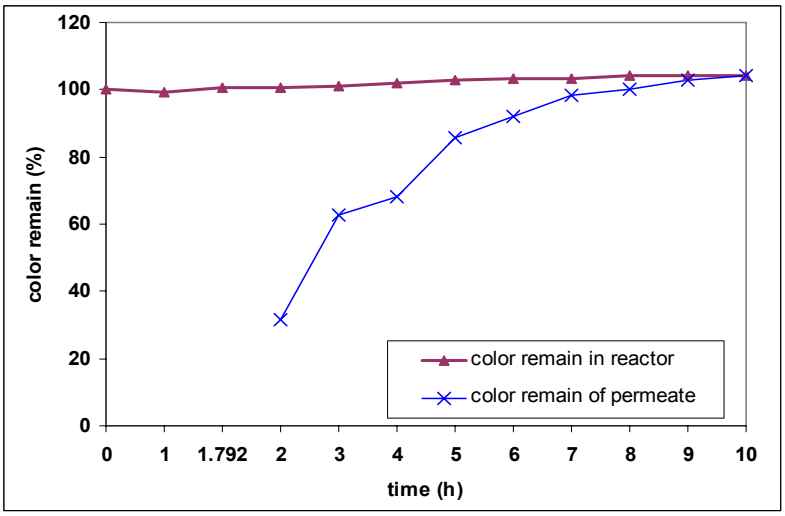


Figure 9.12 Decolorization of viandox with mineral membrane bioreactor using HRT of 40 hours without bacterial culture.

COD removal efficiency

The variation of supernatant inside reactor and effluent COD concentration with time during the experiment is illustrated in Figure 9.13. The COD of supernatant in reactor and permeate after operation for 10 days remained at about 22,000 mg/L and 20,470 mg/L, respectively, although the influent COD was around 22,000 mg/L. Thus, the average removal of COD was 9.09%.

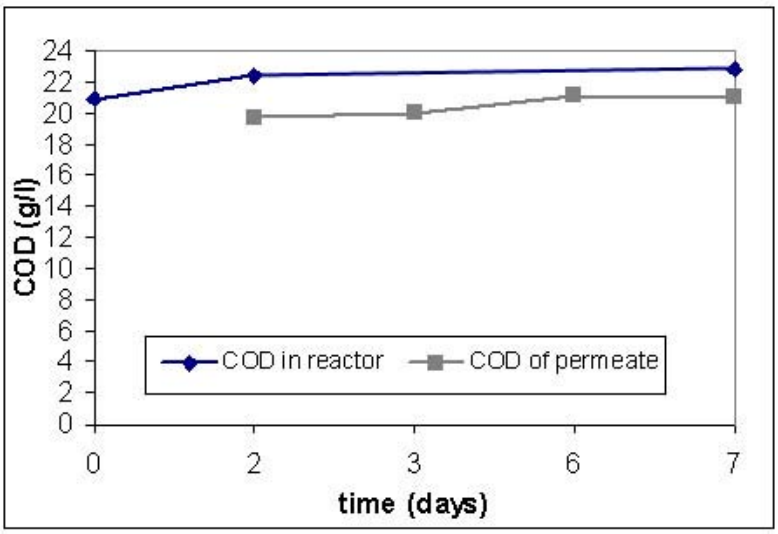


Figure 9.13 Variation of COD in bioreactor and permeate with time in mineral membrane bioreactor which using a HRT of 40 hours without bacterial culture.

Membrane permeability

Figure 9.14 shows the variation of permeability of the mineral membrane before and after operation with HRT of 40 h. The permeability of mineral membrane after 10 days of operation was equal to permeability before being used. It could be concluded that irreversible (adsorption) membrane fouling did not occur under this operating condition.

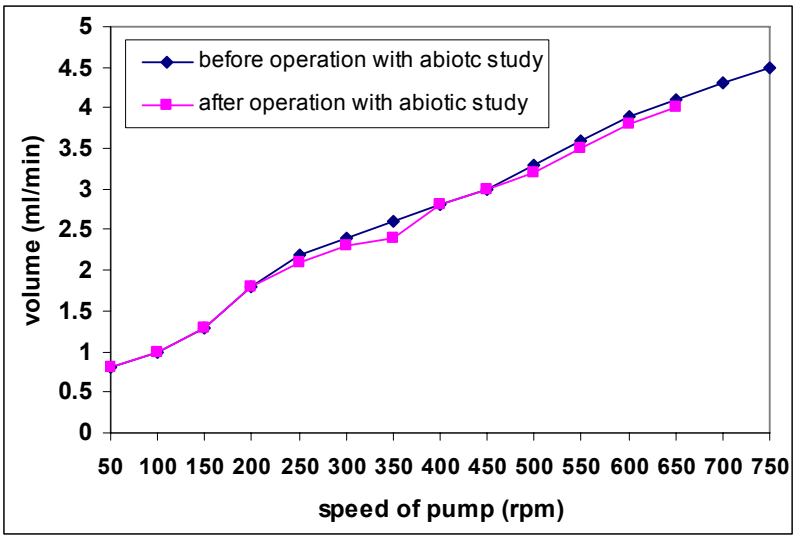


Figure 9.14 Variation of permeability of the mineral membrane before and after 50 days of operation with HRT of 40 hours without bacterial culture.

9.3.3 Decolorization experiment using mineral MBR at HRT of 20 hours.

Color variation with time

The MBR performance was initially design with HRT of 20 hours (Figure 9.15-9.16). Figure 9.15 shows the evolution of biomass and color concentrations during 13 days of operation. The variation of permeate and feed color with time is shown in Figure 9.16. The results showed that the residual color of the permeate was consistently lower than the sludge supernatant color inside membrane bioreactor. Maximum decolorization of 51.18 % was observed in 2 days and the color rather increased until 6 days. Then, the bacterial consortium in the system could decolorize melanoidins again and later reached steady decolorization without significant color reduction. After operation for 13 days, the decolorizing activity of bacterial consortium was constant approximately 20 %. These results are quite similar to the previous results at HRT=40 hours, but in lower color removal percentage, due to the lower accumulation effect, itself linked to the reduced HRT.

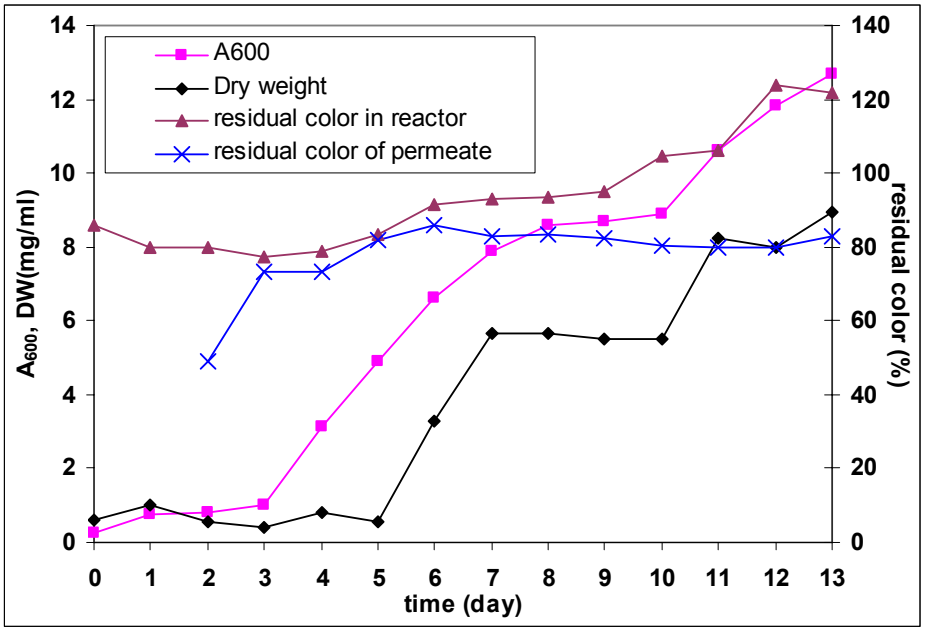


Figure 9.15 Time course of decolorization of the synthetic melanoidins-containing wastewater medium by the bacterial consortium MMP1 in mineral membrane bioreactor using HRT of 20 hours. The consortium was cultured under the condition described in the text. Symbols: (▲), residual color of culture medium in bioreactor, (**x**) residual color of culture medium in permeate, (♦) bacterial dry weight and, (■) optical density at 600 nm.

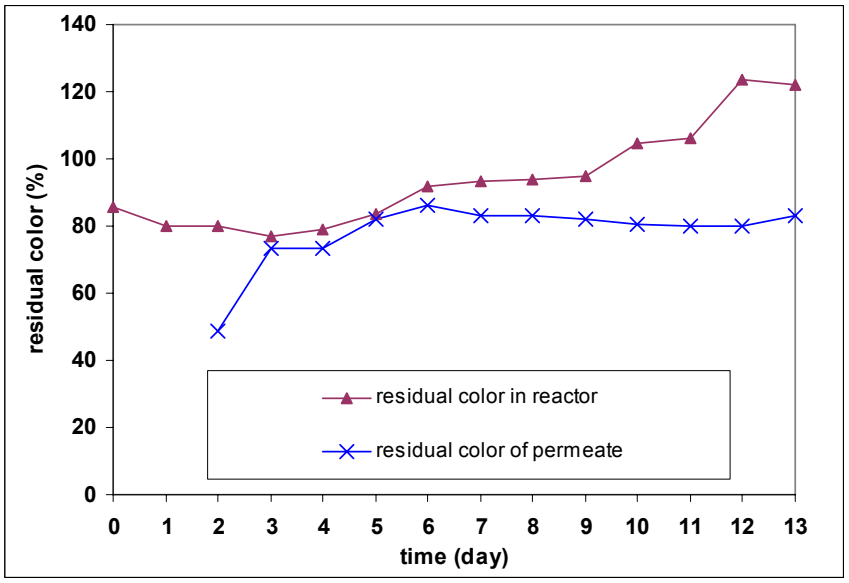


Figure 9.16 Variation of permeate and feed color with time in mineral membrane bioreactor using HRT of 20 hours.

Sludge production

The growth of bacterial consortium MMP1 during the experiment is shown in Figure 9.17. Time course of effluent decolorization was studied along with the growth of the consortium MMP1. With the increase of time, there was an increase in cells number. After each decline, the biomass was stabilized; this can be explained by the adaptation of microorganisms in membrane reactor (Badani et al., 2005). The colonies and microscopic photograph of bacterial consortium MMP1 in mineral membrane bioreactor operated with HRT of 20 hours were showed in Figure 9.18.

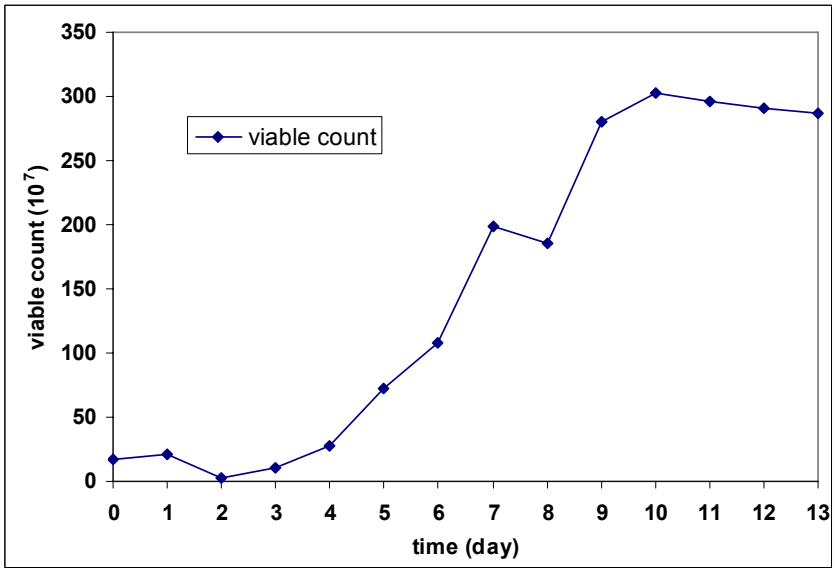


Figure 9.17 Time course of bacterial growth in the synthetic melanoidins-containing wastewater medium in mineral membrane bioreactor using HRT of 20 hours.

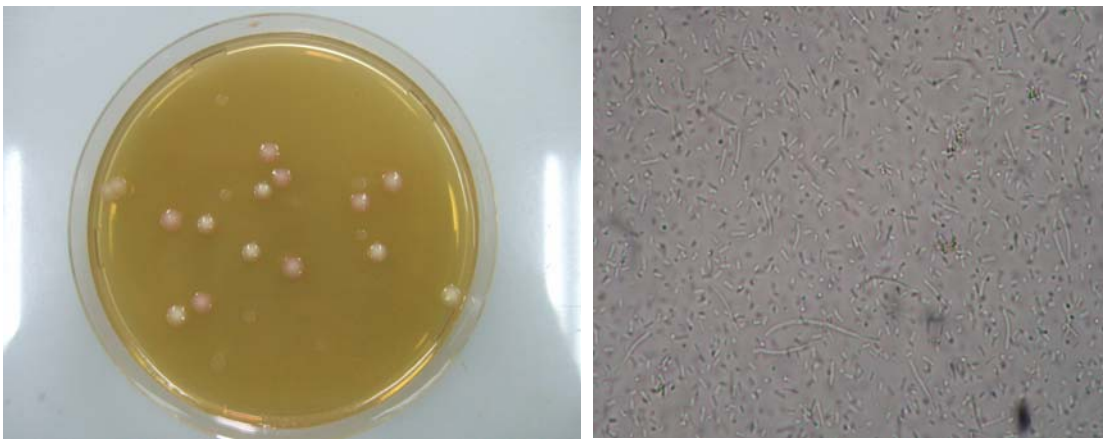


Figure 9.18 Colonies and microscopic photograph of bacterial consortium MMP1 in mineral membrane bioreactor using HRT of 20 hours.

COD removal efficiency

The variation of influent and effluent COD concentration with time during the experiment is illustrated in Figure 9.19. The COD of permeate after 13 operation days remained at about 17,632 mg/L although the influent COD was around 22,000 mg/L. Thus, the average removal of COD was 19.86%.

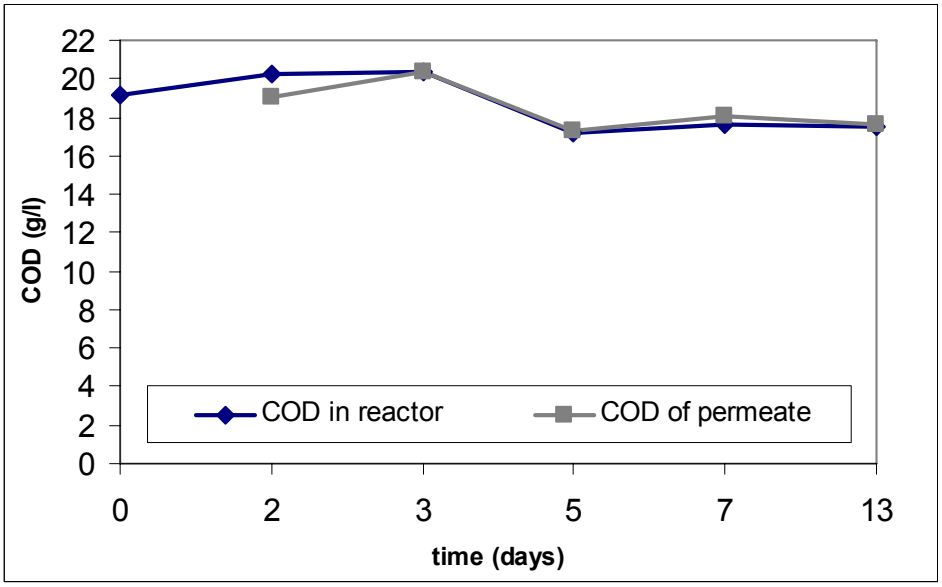


Figure 9.19 Variation of COD in bioreactor and permeate with time in mineral membrane bioreactor using HRT of 20 hours.

Membrane permeability

Figure 9.20 shows the variation of permeability of the mineral membrane before and after operation with HRT of 20 hours. The permeability of mineral membrane was dropped after 13 days of operation. It has been observed in Figure 9.11 for a velocity higher than 500 rpm that the difference in permeabilities before and after experiments is more probably due to some dirty particles present in the water, more than to a real irreversible fouling state of the membrane. Thus, in this study, it could be assumed that there is no fouling due to irreversible phenomena. Then, the problem observed on permeate, leading to stop the experiment is due to a too weak selected cross flow velocity, or more probably to a clogging of the membrane pores by the development of thick biofilm, as previously observed with the hollow fibers membranes.

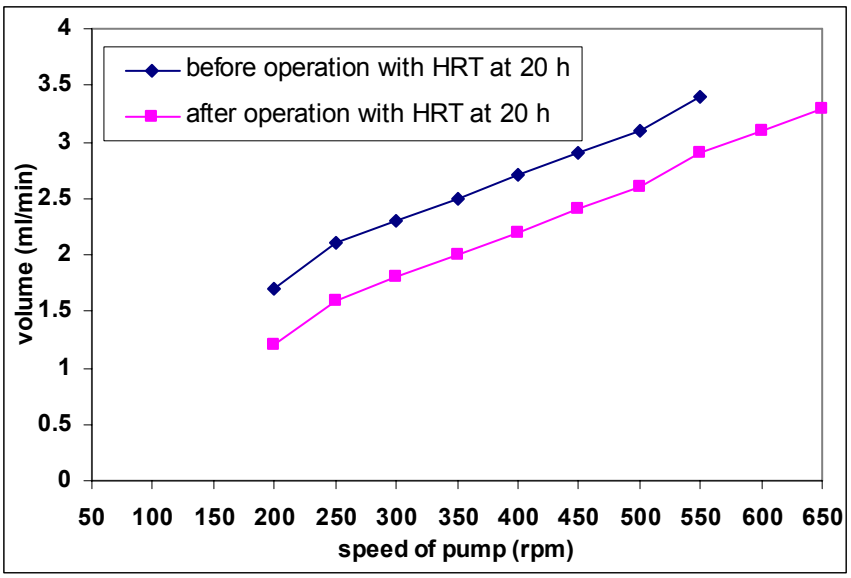


Figure 9.20 Variation of permeability of the mineral membrane before and after 13 days of operation with HRT of 20 hours.

9.3.4 Decolorization experiment using mineral MBR at HRT of 15 hours

Color variation with time

In the present study, MBR performance was investigated with a HRT of 15 hours (Figure 9.21- 9.22). The variation of permeate and feed color with time is shown in Figure 9.22. The results showed that the residual color of an effluent was consistently lower than the sludge supernatant inside membrane bioreactor. Maximum decolorization of 50.44 % was observed in first day and then the color rather increased in the second day. After 4 days of operation, the decolorizing activity of bacterial consortium was constant approximately 23.05 %. Unfortunately, the MBR was stopped after operation for 4 days since no more permeate was flowing. Permeate was slowly stopped and the supernatant in the reactor was increased since the membrane was fouling and became clogged.

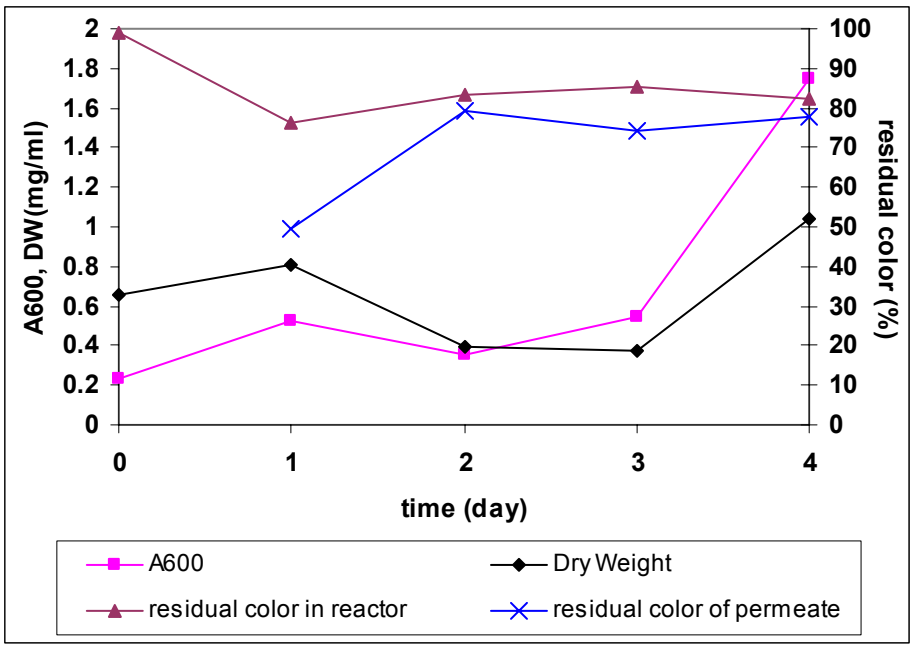


Figure 9.21 Time course of decolorization of the synthetic melanoidins-containing wastewater medium by the bacterial consortium MMP1 in mineral membrane bioreactor using HRT of 15 hours. The consortium was cultured under the conditions described in the text. Symbols: (▲), residual color of culture medium in bioreactor, (**x**) residual color of culture medium in permeate, (♦) bacterial dry weight and, (■) optical density at 600 nm.

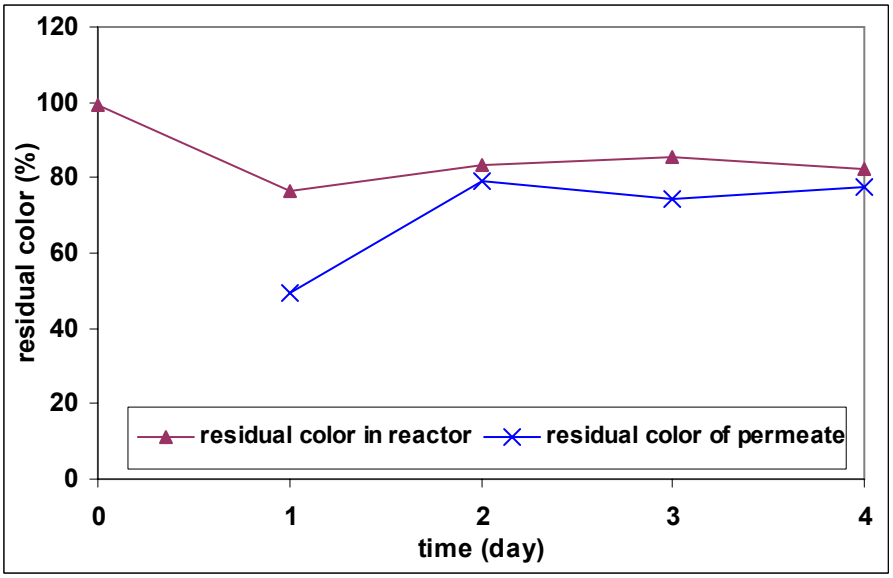


Figure 9.22 Variation of permeate and feed color with time in mineral membrane bioreactor using HRT of 15 hours.

Sludge production

Figure 9.23 showed the evolution of biomass concentration and color concentration during the 4 days of operation. The growth of bacterial consortium MMP1 during the experiment is shown in Figure 9.21. Time course of effluent decolorization was studied along with the growth of the consortium MMP1. The fluctuation of the bacterial number within the first 4 days was assumed that the bacterial consortium MMP1 was acclimatized to the culture condition within MBRs. Unfortunately, system has failed with a membrane fouled and completely clogged. In the present study, as with the others HRT a lag phase of 4 days was observed.

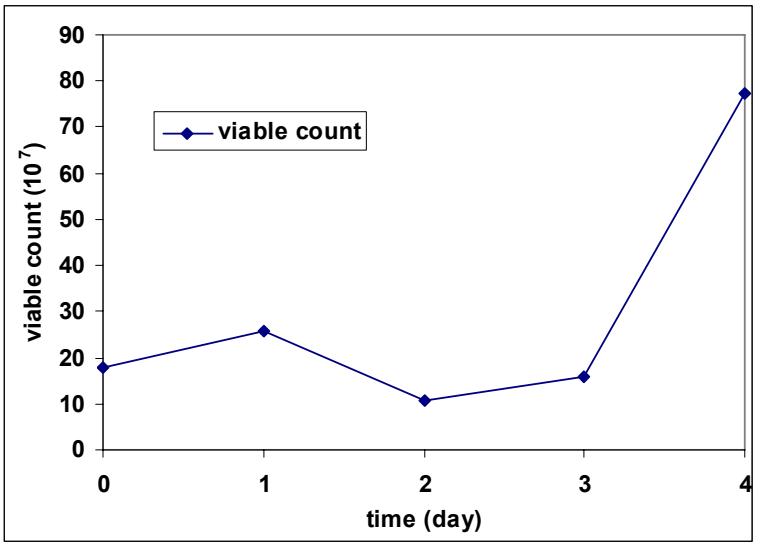


Figure 9.23 Time course of bacterial growth in the synthetic melanoidins-containing wastewater medium in mineral membrane bioreactor using HRT of 15 hours.

COD removal efficiency

The variation of influent and effluent COD concentration with time is illustrated in Figure 9.24. The COD of permeate after 4 operation days remained at about 19,757 mg/L although the influent COD was around 22,000 mg/L. Thus, the average removal of COD was 10.19%. Due to the bacterial cells in bioreactor after 4 days of operation were still in lag-phase thus, the COD removal efficiency in the MBR with 15-hour HRT was lower than the higher HRT.

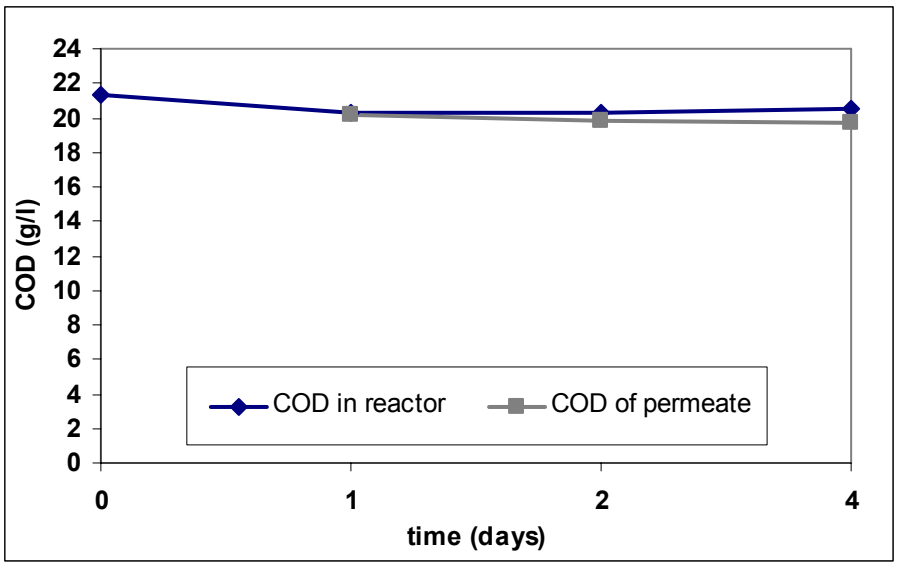


Figure 9.24 Variation of COD in bioreactor and permeate with time in mineral membrane bioreactor using HRT of 15 hours.

Membrane permeability

Figure 9.25 shows the variation of permeability of the mineral membrane before and after operation with the HRT of 15 hours. The permeability of the membrane was dropped down after 4 days of operation then, completely clogged and no more permeate was observed. It could be concluded that membrane fouling occurred under this operating conditions. Also, it can be observed in Figure 9.25 that washing the membrane with cleaning agents (diluted NaOH and HNO_3) let increasing the permeability of the used membrane until its original values. Hong et al. (2002) mentioned that permeate flux decline due to irreversible fouling can be recovered by chemical cleaning and mechanical backwashing, that have been done here.

Membrane fouling is often considered to be caused by the deposition of particles on the membrane surfaces. Colloidal particles were identified as a predominant factor controlling membrane permeability, even though they make up a very small fraction of the total particles in MBR systems. In addition, Chang and Kim had found that small colloidal particles might play a critical role in membrane fouling in MBR systems (Chang and Kim, 2005). Because extracellular polymeric substances (EPS) are one of the main components of colloidal particles in MBR wastewater treatment systems,

Based on the above information and results in this study, it is possible that the formation of colloidal compounds and dissolved organic compounds in bioreactors might be related to membrane fouling.

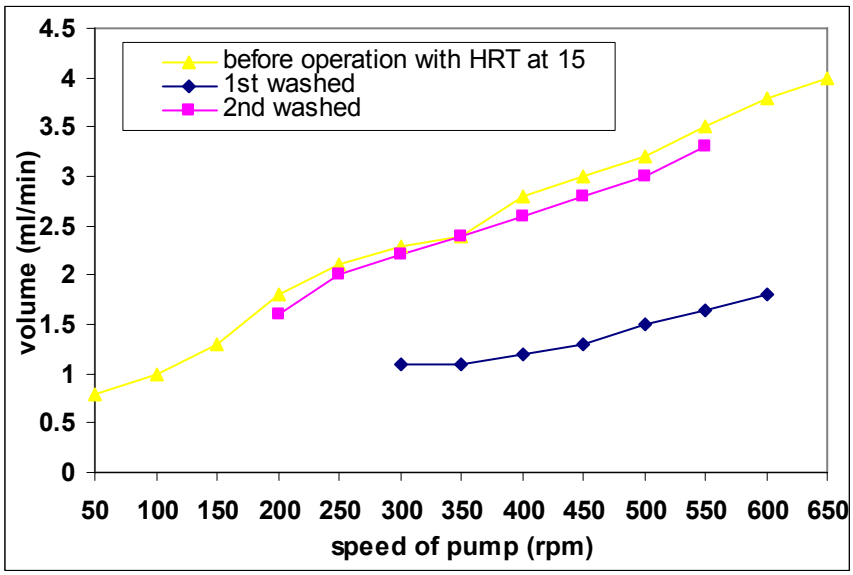


Figure 9.25 Variation in permeability of the mineral membrane before and after 4 days of operation with the HRT at 15 hours.

The MBR process was found to be useful when a long solid retention time is required and physical retention and subsequent microbial activity in MBR are maximized (Knoblock et al., 1994). This process provides benefits over conventional biological wastewater treatment in terms of high effluent quality, reduced sludge wasting and production and improved biological degradation. Along with biological degradation, the membrane itself physically removes some of the contaminants (Klatt and LaPara, 2003).

In this study, the performance of a laboratory-scale side-stream bioreactor treating the synthetic melanoidins-containing wastewater was investigated. The efficiency of membrane coupled bioreactor systems at different hydraulic retention times of 40, 20 and 15 hours was studied some-days experiments. The study showed that COD, as well as color removal efficiency were dependent on HRT. The operation at longer HRT has shown a slight benefits for treatment of the synthetic melanoidins-containing wastewater. In any case, the HRT must be higher than 20 hours otherwise clogging may occur rapidly.

Unfortunately, because of time spent for each experiment, it had not enough time to try again the same biological conditions with different hydraulic conditions, specially the cross-flow velocity in the tubular membrane. It seems that if some fouling occurred, it is not only irreversible so that tangential velocity should limit it. Moreover it would be convenient to test a device with the ability to measure and increase the transmembrane pressure.

Hydraulic retention time (HRT) plays an important role in the removal of pollutants in conventional bioreactors. In case of MBRs, it is a common observation that with the increase of HRT, some pollutants, the removal efficiency of the system increases (Tay et al., 2003). This retention increases residence time and allows the biomass to degrade these recalcitrant compounds. Unfortunately, these same compounds are thought to lead to membrane fouling. Similarly, the present study shows significant improvement in COD and color removal efficiency were observed with the longer HRT. Moreover, the increase of HRT showed a prolonging of membrane fouling limitation.

Solid retention time (SRT) is one of the most critical parameter for MBRs design as SRT affects the treatment process performance, aeration tank volume, sludge production, and oxygen requirements. The longer retention time of all biomass within the MBR over the conventional biological wastewater treatment allowed for the bacterial consortium MMP1 to acclimatize and sustain its decolorization in the MBR conditions. However, in this study, the color and COD removal efficiency of the synthetic melanoidins-containing wastewater was relatively low due to the presence of recalcitrant organics and growth inhibiting substances, which may be retained by the membrane. Furthermore, COD removal efficiency was not significantly affected by the increase in bacterial concentration. These results showed that for the chosen conditions, the color concentration of treated water requires a post-processing wastewater treatment system in order to eliminate the remaining color, but more probably an optimization of the operating conditions of the MBR will lead to more convenient results, especially a real acclimatization of the biomass in MBR for 2 or 3 times the SRT..

Membrane fouling depends on the characteristics of membrane materials. In this study, fouling of membranes occurred with the experiments of polysulfone hollow-fiber membrane and tubular membrane with HRT of 15 h. Although it is difficult to establish a general rule about membrane fouling in MBRs, fouling are strongly influenced by three factors: membrane characteristics, operating conditions and biomass characteristics (Le-Clech, 2003). However, chemical cleaning of the

membrane with the dilute bases and acidic detergents was found to produce an acceptable effect in terms of cleaning time and recovered flux.

CHAPTER X

CONCLUSIONS

Molasses-based distilleries are one of the most polluting industries generating large volume of colored wastewater. Thus, the aim of this dissertation was to explore the feasibility of using bacterial isolates for decolorization of the synthetic molasses-containing wastewater, with the ultimate aim of application for molasses-based distillery wastewater treatment. In this work, a bacterial consortium capable of decolorizing molasses wastewater was isolated from various sources in Thailand. Various parameters for maximal molasses wastewater decolorizing activity were also observed.

The present study started with the isolation of various molasses-decolorizing bacterial from different sources. After that, the identification of bacterial cultures by 16S rDNA based molecular approach were carried out. However, it clearly indicated that pure culture of bacterial isolate displayed a limited capability for decolorization of molasses wastewater in long-term treatment and it is necessary to improve its cultured conditions for further decolorization processes. Hence, in the Chapter 4, the molasses decolorizing bacterial consortium was isolated and in the Chapter 6, the artificial bacterial consortium MMP1 comprising of *Klebsiella oxytoca* (T1), *Serratia mercescens* (T2) and unknown bacterium DQ817737 (T4), was constructed. This bacterium consortium exhibited increased decolorization compared to that shown by any single isolate. This may be due to the enhanced effect of coordinated metabolic interactions on melanoidins decolorization. Then, the bacterial consortium MMP1 could be utilized for the decolorization of various kinds of melanoidins present in various industrial effluents including sugarcane and beet molasses wastewaters.

Environmental factors for example pH, temperature, aeration and nutrients play vital role in bacterial degradation process of industrial wastes, as the enzymes activity is greatly influenced by these environmental factors. Several studies have been carried out by groups of scientists to understand the role of various environmental factors in the degradation of melanoidins toward the maximum degradation and decolorization with different microbial species (Mohana et al., 2007; Kumar and Chandra, 2006; Sirianuntapiboon et al., 2004). In this study, the consortium showed the highest growth and melanoidins decolorization at the initial pH of 4 under low aeration condition. Thus, the consortium MMP1 might be suitably applied to the acid formation phase of conventional aerobic or anaerobic treatment systems of alcoholic distillery wastewater.

Also, it has been demonstrated that the aeration condition has a critical effect on melanoidins decolorization. In general, various aerobic bacterial isolates and

consortia that have been shown high melanoidins degradation efficiency are not suitable for treating effluent from molasses-based distillery industries in the absence of aeration. In contrast to other studies, the results presented herein have shown that color removal of the bacterial consortium under facultative and anaerobic conditions were higher than aerobic condition. Hence, the decolorization mechanisms of molasses wastewater by this bacterial consortium might result from 2 possible mechanisms. One was due to the color adsorption by bacterial cells and another might be due to the metabolism of bacterial cells under facultative and anaerobic conditions such as fermentation and anaerobic respiration. In addition, as shown in Chapter 7, the comparison of decolorization of consortium MMP1 with abiotic control has proved that the color removal for synthetic melanoidins-containing wastewater medium containing 2% (v/v) Viandox was due to biotic activity of bacteria but not to adsorption of color substances on cells surface.

Biological treatments employing fungi and bacteria have been investigated essentially to decolorize the distillery spent wash. In all cases, it was found necessary to supplement with additional nutrients as well as diluting the effluent for obtaining optimal microbial activity and eventually optimal results (Ohmomo et al., 1988; Sirianuntapiboon et al., 2004). Adding nutrients to biological treatment processes is one of the possible approaches to upgrading an existing facility in order to deal with increasing volumes and strengths of industrial wastewaters. Thus, the study in Chapter 8 was set up to investigate the limitation of decolorization of the synthetic melanoidins-containing wastewater medium by the constructed bacterial consortium MMP1. The results showed that the addition of nutrients had a favorable effect on decolorization of the used bacterial cells inoculated in fresh medium. This suggested that the decolorization of melanoidins ran parallel with the decomposition of nutrients. Therefore, nutrients could affect the growth and melanoidins decolorization of consortium MMP1. Furthermore, the addition of 0.5% (w/v) LB and B vitamins provided the similar results and their decolorization did not increase significantly relative to the reused culture medium without nutrient supplementation. Therefore, the limitation of decolorization efficiency of the bacterial consortium MMP1 in this study might not directly be due to the nutrient-limitation.

For process application, it was assumed that the constructed bacterial consortium MMP1 had the potential to be used as an inoculum for decolorization of melanoidins-containing wastewaters since its highest decolorization took place under the condition similar to the real distillery wastewaters. Thus, the laboratory-scale non-immersed bioreactor was designed, built and evaluated for the treatment the synthetic melanoidins-containing wastewater in the Chapter 9. In this context, a feasible system may be envisaged by coupling the degradation capability of bacterial

consortium MMP1 with inherent advantages of membrane bioreactor. The results obtained from membrane coupled bioreactor systems at different hydraulic retention times (HRT) of 40, 20 and 15 h indicated that COD removal efficiency was dependent of HRT. The operation with longer HRT has shown benefits for treatment of the synthetic melanoidins-containing wastewater. In general, bacterial concentration is an important parameter in performance as it represents the biomass concentration in the reactor; also increasing bacterial concentration implies increasing COD removal rate. In contrast, in this study, COD removal efficiency was not significantly affected by the increase in bacterial concentration. Moreover, the color and COD removal efficiency of the synthetic melanoidins-containing wastewater was relatively low due to the presence of recalcitrant organics and growth inhibiting substances (Satyawali and Balakrishnan, 2008; Chandra et al., 2008). The performance of a laboratory-scale MBR used in this study was compared with the existing wastewater treatment system as shown in Table 10.1. These results show that the color concentration of treated water still requires a post-processing wastewater treatment system in order to eliminate the remaining color, or a better understanding of the MBR operating conditions influence on the performance of the process.

Table 10.1 Performance of various membrane reactors for molasses distillery wastewater

Wastewaters MBR	Configuration	Materials	HRT	SRT	COD removal	Color removal	References
Shochu distillery wastewater	External MBR (EMBR)	Polysulfone molecular waight cut off 2,000 KDa.	NR	No discharged	98%	NR	Nagano et al., 1992
Brewery wastewater	Anaerobic digestion Ultrafiltration (ADUF)	Polyethersulfone 0.44 µm	19.2 h	NR	97%	NR	Ross and Strohwald, 1994
Wine Distillery	Anaerobic digestion Ultrafiltration (ADUF)	Polyethersulfone 0.44 µm	72 h	NR	93%	NR	
Simulated distillery wastewater	Submerged MBR (SMBR)	0.2 µm stainless steel	10 –30 h	NR	94.7%	NR	Zhang et al., 2006
Distillery wastewater	External MBR (EMBR) Hybrid nanofiltration Nanofiltration (NF) and Reverse Osmosis process	Commercial Nanofiltration and thin film composite polyamide RO in spiral wound	NR	NR	99.9%	80%	Nataraj et al., 2006
Synthetic melanoidins-containing wastewater	Side-stream MBR	0.14 µm mineral membrane (M14 Carbosep®)	40 h	50 days	25.5%	13.5%	This study

NR: Not reported

Recommendations for future molasses wastewater treatment

Considering the decolorization performances and COD removal efficiency of the bacterial consortium MMP1, biological treatment coupled with membrane technology cannot be absolute treatment of molasses-based distillery wastewater. Hence, there is a need to establish a comprehensive treatment approach involving all the technologies sequentially. Whatever the methods employed for treatment of effluent from molasses-based distillery industries, it can be concluded that in an ideal cost objective, the commercial treatment scheme should comprise a biological processes as the primary step followed by a physicochemical treatment.

The following recommendations are intended to provide insight into the molasses-based distillery wastewater treatment process to achieve consistent and high quality effluent suitable for environmental discharge.

- It is established that several microorganisms such as bacteria and fungi, especially in pure cultures, show a limited ability to decolorize the molasses-based distillery. Thus, a better understanding of the microbial activities responsible for the degradation of melanoidins and the interaction between pure cultures in the consortium would contribute to enhancing the efficiency of the overall treatment system.

- Most of the investigations on bacterial isolates that can result in both color and COD removal have been limited exclusively in laboratory scale experiment. The issues of appropriate system design and scale-up have rarely been addressed. In this context, treatment systems like membrane bioreactors that have lower sludge production can be considered. In addition, the minimizing of nutrient supplementation should be examined. These issues are particularly significant in the application field of molasses-based distillery wastewater treatment.

- The operations of membrane bioreactor for treatment of molasses-based distillery wastewaters are characterized by significant membrane fouling that limits its applicability. Membrane bioreactor can result in significant color removal. However, molasses distillery is a complex and multi-components wastewater that is known to cause membrane fouling. Moreover, the characteristics and structure of melanoidins (color-causing substances in molasses distillery) is still not fully understood. Thus, the understanding of the wastewater components which are primarily responsible for this phenomenon would contribute to an appropriate and efficient usage of the membrane bioreactor. In addition, the future trends in MBR process optimization would

involve the modeling of parameters affecting the performance of wastewater treatment.

REFERENCES

Acharya, B.K., Mohana, S., and Madamwar, D. 2008. Anaerobic treatment of distillery spentwash: A study on upflow anaerobic fixed film bioreactor. Bioresource Technology 99: 4621-4626.

Alfafara, C.G., Migo, V.P., Amrante, J.A., Dallo, R.F., and Matsumara, M. 2000. Ozone treatment of distillery slop waste. Water Science and Technology 42 (3-4): 193-198.

Alkane, H.V., Dange, M.N., and Selvakumari, K. 2006. Optimization of anaerobically digested distillery molasses spent wash decolorization using soil as inoculum in the absence of additional carbon and nitrogen source. Bioresource Technology 97: 2131-2135.

Akunna, J.C., and Clark, M. 2000. Performance of a granular-bed anaerobic baffled reactor (GRABBR) treating whisky distillery wastewater. Bioresource Technology 74: 257-261.

Ames, M.J., Wynne, A., Hofmann, A., Plos, S., and Gibson, R.G. 1999. The effect of a model melanoidin mixture on faecal bacterial populations in vitro. British Journal of Nutrition 82: 489-495.

American Public Health Association (APHA). 1998. Water Pollution Control Federation. In American Water Works Association (20th ed.), Standard Methods for the Examination of Water and Wastewater, Washington, DC: American Public Health Association.

Angayarkanni, J., Palaniswamy, M., and Swaminathan, K. 2003. Biotreatment of distillery effluent using *Aspergillus niveus*. Bulletin of Environmental Contamination and Toxicology 70: 268-277.

Asthana, A., Mishra, S.K., Chandra, R., and Guru, R. 2001. Treatment of colour and biochemical oxygen demand of anaerobically treated distillery effluent by aerobic bacterial strains. Indian Journal of Environmental Protection 21: 1070-1072.

Badani, Z., Ait-Amar, H., Si-Salah, A., Brik, M., and Fuchs, W. 2005. Treatment of textile wastewater by membrane bioreactor and reuse. Desalination 185: 411-417.

Bailey A. D., Hansford G. S., and Dold P. L. 1994. The enhancement of upflow anaerobic sludge bed reactor performance using crossflow microfiltration. Water Research 28: 291-295.

Banerjee, S., and Biswas, G.K. 2004. Studies on biomethanation of distillery wastes and its mathematical analysis. Chemical Engineering Journal 102: 193-201

BCC Research. 2008. Forecasting the global market value of membrane bioreactor [online]. Available from: www.bccresearch.com [2008, October 10]

Beltran, F.J., Encinar, J.M., and Gonza´ lez, J.F. 1997. Industrial wastewater advanced oxidation. Part 2. Ozone combination with hydrogen peroxide or UV radiation. Water Research 31(10): 2415-2428.

Benito, G.G., Miranda, M.P., and Santos, D.R. 1997. Decolourization of wastewater from an alcoholic fermentation process with Trametes versicolor. Bioresource Technology 61: 33-37.

Bernardo, E.C., Egashira, R., and Kawasaki, J. 1997. Decolorization of molasses wastewater using activated carbon prepared from cane bagasses. Carbon 35: 1217-1221.

Bes-Pia, A., Mendoza-Roca, J.A., Alcaina-Miranda, M.I., Iborra-Clar, A., and Iborra-Clar, M.I. 2003. Combination of physico-chemical treatment and nanofiltration to reuse wastewater of printing, dyeing and finishing textile industry. Desalination 157: 73-80.

Beveridge, T.J. 2005. Bacterial wall structure and implications for interaction with metal ions and minerals. Journal of Nuclear and Radiochemical Sciences 6: 7-10.

Bhandari, H.C., Mitra, A.K., and Kumar, S. 2004. Crest's integrated system: reduction and recycling of effluents in distilleries. In Tewari, P.K. (ed.), Liquid Asset, Proceedings of Indo-EU Workshop on Promoting Efficient Water Use in Agro-based Industries, pp. 167-169. New Delhi, India: TERI Press.

Billore, S.K., and others. 2001. Treatment of a molasses based distillery effluent in a constructed wetland in central India. Water Science and Technology 44: 441-448.

Boria, R., Sanchez, E., Marin, A., and Jimenez, A.M. 1996. Kinetic behavior of waste tyre rubber as microorganism support in an anaerobic digester treating cane molasses distillery slops. Bioprocess and biosystems engineering 16: 17-23

Box, G.E.P., Hunter, W.G., and Hunter, J.S. 1978. Statistics for experimenters. New York: Wiley

Brindle, K., and Stephenson, T. 1996. The application of membrane biological reactors for the treatment of wastewater. Biotechnology and Bioengineering 49: 601-610.

Burgess, J. E., Quarmby, J., and Stephenson, T. 1999. The role of micronutrients in biological treatment of industrial effluents using the activated sludge process. Biotechnology Advances 17: 49-70.

Cämmerer, B., Jalyschkov, V., and Kroh, L.W. 2002. Carbohydrate structures as part of the melanoidin skeleton. International Congress Series 1245**:** 269-273.

Cämmerer, B., and Kroh, L.W. 1995. Investigations of the influence of reaction conditions on the elementary composition of melanoidins. Food Chemistry 53: 55-59.

Chandra, R., Bharagava, N., and Rai, V. 2008b. Melanoidins as major colourant in sugarcane molasses based distillery effluent and its degradation. Bioresource Technology 99 (11): 4648-4660.

Chandra, R., Yadav, S., Bharagava R.N., and Murthy, R.C. 2008a. Bacterial pretreatment enhances removal of heavy metals during treatment of post-methanated distillery effluent by *Typha angustata* L. Journal of Environmental Management 88: 1016-1024.

Chandraraj, K., and Gunasekaran, P. 2004. Bacterial alcoholic fermentation. In Pandey, A. (ed.), Concise Encyclopedia of Bioresource Technology, pp. 327-333. New York: Food Products Press.

Chang, I.S., Choo, K.H., Lee, C.H.. Pek, U.H., Koh, U.C., Kim, S.W., and Koh, J.H. 1994. Application of ceramic membrane as a pre-treatment in anaerobic digestion of alcohol-distillery wastes. Journal of Membrane Science 90 (1-2): 131-139.

Chang, W.K., Hu, A.Y.J., Horng, R.Y., and Tzou, W.Y. 2007. Membrane bioreactor with nonwoven fabrics as solid-liquid separation media for wastewater treatment. Desalination 202: 122-128.

Chaturvedi, S., Chandra, R., and Rai, V. 2006. Isolation and characterization of *Phragmites australis* (L.) rhizosphere bacteria from contaminated site for bioremediation of colored distillery effluent. Ecological Engineering 27: 202-207.

Chaudhari, P.K., Mishra, I.M., and Chand, S. 2008. Effluent treatment for alcohol distillery: Catalytic thermal pretreatment (Catalytic thermolysis) with energy recovery. Chemical Engineering Journal 136: 14-24.

Chen, J.C.P., and Chou, C.C. 1993. Cane Sugar Handbook, pp. 408-409. New York: Wiley.

Choo, K.H., and Lee, C.H. 1996. Membrane fouling mechanisms in the membrane coupled anaerobic bioreactor. Water Research 30(8): 1771-1780.

Cindy, H. N., Vigdis, T., and Lose, O. 2000. Soil community analysis using DGGE of 16S rDNA polymerase chain reaction products. Soil Science Society of America Journal 64: 1382-1388.

Coca, M., Pena, M., and Gonzalez, G. 2005. Chemical oxidation processes for decolorization of brown-colored molasses wastewater. Ozone Science & Engineering 27: 365–369.

Coca, M., Teresa Garc a , M., Gonzalez, G., Pena, M., and Garc a, J.A. 2004. Study of coloured components formed in sugar beet processing. Food Chemistry 86: 421-433.

Cortez, L.A.B., Pere´ z, L.E.B. 1997. Experiences on vinasse disposal: Part III: combustion of vinasse-]6 fuel oil emulsions. Brazilian Journal of Chemical Engineering [online]. Available from: http://www.scielo.br/scielo.php?script

Cox, D.R. 1958. Planning of experiments. New York: Wiley

Dahiya, J., Singh, D., and Nigam, P. 2001b. Decolorization of molasses wastewater by cells of *Pseudomonas fluorescens* immobilized on porous cellulose carrier. Bioresource Technology 78: 111-114.

Department of Industrial Works. 1992. The Standard of Industrail Effluent Quality Due to the Ministry of Science Technology and Environment's regulations, pp. 1-55. Bangkok, Thailand: Department of Industrial Works, Ministry of Industry.

De Wilde, F.G.N. 1987. Demineralization of a molasses distillery wastewater. Desalination 67, 481-493.

Doyen, W., Adriansens, W., Molenberghs, B., and Leysen, R. 1996. A comparison between polysulfone, zirconia and organo-mineral membranes for use in ultrafiltration. Journal of Membrane Science 113: 247-258.

Duckworth, W.E. 1968. Statistical techniques in technological research. London: Methuen.

D'souza, D.T., Tiwari, R., Sah, A.K., and Raghukumar, C. 2006. Enhanced production of Laccase by a marine fungus during treatment of coloured effluents and synthetic dyes. Enzyme and Microbial Technology 38: 504-511.

Eccles, H. 1995. Removal of heavy metals from effluent streams why select a biological process. International Biodeterioration and Biodegradation 35: 5-16.

Ecologix Environmental Systems, LLC. 2008. Coagulation and Flocculation [online]. Available from: www.ecologixsystems.com [2008 February 10]

Edenborn, S. L., and Sexstone, A. J. 2007. DGGE fingerprinting of culturable soil bacterial communities complements culture-independent analyses. Soil Biology and Biochemistry 39: 1570-1579.

Fahy, V., FitzGibbon, F.J., McMullan, G., Singh, D., and Marchant, R. 1997. Decolorization of molasses spent wash by Phanerochaete chrysosporium. Biotechnology Letters 19: 97-99.

Fan, B., and Huang, X. 2002. Characteristics of a self-forming dynamic membrane coupled with a bioreactor for municipal wastewater treatment. Environmental Science and Technology 36: 5245-5251.

Fang, H.H.P., Chui, H.K., and Li, Y.Y. 1994. Microbial structure and activity of UASB granules treating different wastewaters. Water Science and Technology 30: 87-96.

Fay, L.B., and Brevard, H. 2004. Contribution of mass spectrometry to the study of the Maillard reaction in food. Mass Spectrometry Reviews 24(4): 487-507.

Friedrich, J. 2004. Bioconversion of distillery waste. In Arora, D.K. (ed.), Fungal biotechnology in agriculture, food and environmental applications, pp. 431-442. New York: Marcel Dekker.

Fuchs, W., Resch, C., Kernstock, M., Mayer, M., Schoeberl, P., and Braun, R. 2005. Influence of operational conditions on the performance of a mesh filter activated sludge process. Water Research 39: 803-810.

Fujita, M., Era, A., Ike, M., Soda, S., Miyata, N., and Hirao, T. 2000. Decolourization of heat-treatment liquor of waste sludge using polyurethane foam-immobilized white rot fungus equipped with an ultramembrane filtration unit. Journal of Bioscience and Bioengineering 90: 387-394.

Gagosian, R.B., and Lee, C. 1981. Process controlling the distribution of biogenic organic compounds in seawater. In Duursma, E.K. and Dawson, R. (eds.), Marine organic chemistry, pp. 91-118. Amsterdam: Elsevier.

Ghosh, M., Ganguli, A., and Tripathi, A.K. 2002. Treatment of anaerobically digested distillery spentwash in a two-stage bioreactor using *Pseudomonas putida* and *Aeromonas* sp. Process Biochemistry 7: 857-862.

Ghosh, M., Verma, S.C., Mengoni, A., and Tripathi, A.K. 2004. Enrichment and identification of bacteria capable of reducing chemical oxygen demand of anaerobically treated molasses spent wash. Journal of Applied Microbiology 96: 1278-1286.

Godbole, J. 2002. Ethanol from cane molasses, Fuel Ethanol Workshop, Honululu, Hawaii [online]. Available from: http://www.hawaii.gov/dbedt/ert/new-fuel/files/ethanol-workshop/10-Godbole-DOE-HI.pdf [2007, August 20]

Godshall, M.A. 1999. Removal of colorants and polysaccharides and the quality of white sugar. In Proceedings of sixth International Symposium Organized by Association Andrew van Hook (AvH), pp 28-35. France: Reims.

Gomaa, O., Abdel, K.H., Mattar, Z., and Hassanein, H. 2003. Decolorization of molasses waste water using *Aspergillus niger*. Egyptian Journal of Biotechnology 13:15-28.

Gonzalez, T., Terron, M.C., Yague, S.; Zapico, E., Galletti, G.C., and Gonzalez, A.E. 2000. Pyrolysis/gas chromatography/ mass spectrometry monitoring of fungal biotreated distillery wastewater using *Trametes* sp. I62 (CECT 20197). Rapid Communications in Mass Spectrometry 14: 1417-1424.

Goodwin, J.A.S., and Stuart, J.B. 1994. Anaerobic digestion of malt whisky distillery potale using upflow anaerobic sludge blanket reactor. Bioresource Technology 49: 75-81.

Guimaraes, C., Porto, P., Oliveira, R., and Mota, M. 2005. Continuous decolourization of a sugar refinery wastewater in a modified rotating biological

contactor with *Phanerochaete chrysosporium* immobilized on polyurethane foam discs. Process Biochemistry 40: 535-540.

Gulati, N., 2004. Conservation of resources using evaporation and spray drying technology for distillery and paper industries. In Tewari, P.K. (ed.), Liquid Asset, Proceedings of Indo-EU Workshop on Promoting Efficient Water Use in Agro-Based Industries, pp. 163-166. New Delhi, India: TERI Press.

Haas, J.R. 2004. Effects of cultivation conditions on acid-base titration properties of *Shewanella putrefaciens*. Chemical Geology 209: 67-81.

Hammer, M. J. 1991. Water and Wastewater Technology. pp. 50-100. New York: John Wiley and Sons.

Han, S. S., Bae, T.H., Jang, G.G., and Tak, T.M. 2005. Influence of sludge retention time on membrane fouling and bioactivities in membrane bioreactor system. Process Biochemistry 40(7): 2393-2400.

Hayashi, T., and Namiki, M. 1986. Role of sugar fragmentation in an early stage browning of amino-carbonyl reaction of sugar with amino acid. Agricultural Biological Chemistry 50: 1965-1970.

Hayase, F., Sato, M., Tsuchida, H., and Kato, H. 1982. Volatile components formed by thermal degradation of nondialyzable melanoidin prepared from a sugar-butylamine reaction system. Agricultural Biological Chemistry 46: 2987-2996.

Hedges, J.I. 1978. The formation and clay mineral reactions of melanoidins. Geochimica et Cosmochimica Acta 42: 69-76.

Hiramoto, K., Nasuhara, A., Michikoshi, K., Kato, T., and Kikugawa, K. 1997. DNA strand-breaking activity of 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP), a Maillard reaction product of glucose and glycine. Mutation Research 395: 47-56.

Hong, S.P., Bae, T.H., Tak, T.M., Hong, S., Randall, A. 2002. Fouling control in activated sludge submerged hollow fiber membrane bioreactors. Desalination 143: 219-228.

Ikan, R., and others. 1992. Chemical, isotopic, spectroscopic and geochemical aspects of natural and synthetic humic substances. Science of The Total Environment 117-118: 1-12.

Ikan, R., Dorsey, T., and Kaplan, I.R. 1990. Characterization of natural and synthetic humic substances (melanoidins) by stable carbon and nitrogen isotope measurements and elemental compositions. Analytica Chimica Acta 232: 11-18.

Jain, M.K., Bhatnagar, L., and Zeikus, J.G. 1990. Biochemical pathways for methane fermentation and use of granulated biomass for high rate anaerobic digestion. Report International Conference on Biogas: Technologies and Implementation Strategies. January 10-15, Pune, India.

Jain, N., Bhatia, A., Kaushik, R., Kumar, S., Joshi, H.C., and Pathak, H. 2005. Impact of post methanation distillery effluent irrigation on ground water quality. Environmental Monitoring and Assessment 110: 243-255.

Jain, N., Minocha, A.K., and Verma, C.L. 2002. Degradation of predigested distillery effluent by isolated bacterial strains. Indian Journal of Experimental Biology 40: 101-105.

Jain, N., Prajapati, S.K., Minocha, A.K., and Verma, C.L. 2000. Batch studies on the degradation of spentwash from distilleries. Indian Journal of Environmental Protection 21(2): 122-126.

Jensen, S., Daae, F. L., and Torsvik, V. 1998. Diversity in methane enrichments from agricultural soil revealed by DGGE separation of PCR amplified 16S rDNA fragments. FEMS Microbiology Ecology 26: 17-26.

Jimenez, A.M., Borja, R., and Martin, A. 2003. Aerobic-anaerobic biodegradation of beet molasses alcoholic fermentation wastewater. Process Biochemistry 38: 1275-1284.

Judd, S. 2006. The MBR Book: Principles and Applications of Membrane Bioreactors in Water and Wastewater Treatment. Oxford: Elsevier.

Juwarkar, A., and Dutta, S.A. 1990. Impact of distillery effluent application to land on soil microflora. Environmental Monitoring and Assessment 15: 201-210.

Kahraman, S., and Yesilada, O. 2003. Decolourization and bioremediation of molasses wastewater by white-rot fungi in a semi-solid state condition. Folia Microbiology 48: 525-528.

Kalavathi, D.F., Uma, L., and Subramanian, G. 2001. Degradation and metabolization of the pigment-melanoidin in distillery effluent by the marine cyanobacterium *Oscillatoria boryana* BDU 92181. Enzyme and Microbial Technology 29: 246-251.

Kalyuzhnyi, S.V., Gladchenko, M.A., Sklyar, V.I., Kizimenko, Y.S., and Shcherbakov, S.S. 2001. One and two-stage upflow anaerobic sludge-bed reactor pretreatment of winery wastewater at 4-10^{O}C. Biotechnology and Applied Biochemistry 90: 107-124.

Kalyuzhnyi, S.V., Martinez, E., and Martinez, J. 1997. Anaerobic treatment of high strength cheese whey wastewater in laboratory and pilot scale UASB reactors. Bioresource Technology 60: 59-65.

Kambe, T.N., Shimomura, M., Nomura, N.; Chanpornpong, T., and Nakahara, T. 1999. Decolourization of molasses wastewater by Bacillus sp. under thermophilic and anaerobic conditions. Journal of Bioscience and Bioengineering 87: 119-121.

Kang, I.J., Yoon, S.H., and Lee, C.H. 2002. Comparison of the filtration characteristics of organic and inorganic membranes in a membrane-coupled anaerobic bioreactor. Water Research 36: 1803-1813.

Kannan, A., and Upreti, R.K. 2008 Influence of distillery effluent on germination and growth of mung bean (*Vigna radiata*) seeds. Journal of Hazardous Materials 153: 609-615.

Kansal, A., Rajeshwari, K.V., Balakrishnan, M., Lata, K., and Kishore, V.V.N. 1998. Anaerobic digestion technologies for energy recovery from industrial wastewater-a study in Indian context, TERI Information Monitor on Environment Science 3: 67–75.

Kato, H., and Tsuchida, H. 1981. Estimation of melanoidins structure by pyrolysis and oxidation. Progress Food Nutritional Science 5: 147-156.

Kida, K., Morimura, S., Abe, N., and Sonoda, Y. 1995. Biological treatment of Shochu distillery wastewater. Process Biochemistry 30: 125-132

Kim, S.J., and Shoda, M. 1999. Decolorization of molasses and a dye by newly isolated strain of the fungus *Geotrichum candidum* Dec1. Biotechnology and Bioengineering 62:114-119.

Klatt, C., and LaPara, T. 2003. Aerobic biological treatment of synthetic municipal wastewater in membrane-coupled bioreactors. Biotechnology and Bioengineering 82(3): 313-320.

Kleerebezem, R., and Macarie, H. 2003. Treating industrialwastewater: ananerobic digestion comes of age [online]. Available from: www.che.com.

Kulkarni, A.K. 1998. Solar assisted photocatalytic oxidation of distillery waste. Indian Chemical Engineer 40 (2): 169-172.

Kumar, P., and Chandra, R. 2004. Detoxification of distillery effluent through *Bacillus thuringiensis* (MTCC 4714) enhanced phytoremediation potential of *Spirodela polyrrhiza* (L.) Schliden. Bulletin of Environmental Contamination and Toxicology 73: 903-910.

Kumar, P., and Chandra, R. 2006. Decolourisation and detoxification of synthetic molasses melanoidins by individual and mixed cultures of *Bacillus* spp. Bioresource Technology 97(16): 2096-2102.

Kumar, S., and Gopal, K. 2001. Impact of distillery effluent on physiological consequences in the freshwater teleost *Channa punctatus*. Bulletin of Environmental Contamination and Toxicology 66: 617-622.

Kumar, S., Sahay, S.S., and Sinha, M.K. 1995. Bioassay of distillery effluent on Common Guppy, *Lebistes reticulates* (Peter). Bulletin of Environmental Contamination and Toxicology 54: 309-316.

Kumar, V., and others. 1997. Bioremediation and decolorization of anaerobically digested distillery spent wash, Biotechnology Letters 19: 311-313.

Kumar, V., and others. 1998. Decolourization and biodegradation of anaerobically digested sugarcane molasses spent wash effluent from biomethanation plants by white-rot fungi. Process Biochemistry 33: 83-88.

Lalov, I.G., Krysteya, M.A., and Phelouzat, J.L. 2001. Improvement of biogas production from vinasses via covalently immobilized methanogens. Bioresource Technology 79: 83-85.

Larter, S.R., and Douglas, A.G. 1980. Melanoidins kerogen precursors and geochemical lipid sinks: a study using pyrolysis gas chromatography (PGC). Geochimica et Cosmochimica Acta 44(12): 2087-2095

Le Clech, P., Jefferson, B., Chang, I.S., and Judd, S.J. 2003. Critical flux determination by the flux-step method in a submerged membrane bioreactor. Journal of Membrane Science 227: 81-93.

Lee, W., Kang, S., and Shin, H. 2003. Sludge characteristics and their contribution to microfiltration in submerged membrane bioreactors. Journal of Membrane Science 216: 217-227.

Lemmer, H., Lind, G., Metzner, G., Nitschke, L., and Schade, M. 1998. Vitamin addition in biological waste- water treatment. Water Science Technology 37: 395-398.

Lettinga, G., and Hulshoff Pol, L.W. 1991. UASB process design for various types of wastewaters. Water Science and Technology 24: 87-107.

Li A. Y., Kothari D., and Corrado J. J. 1984. Application of membrane anaerobic reactor system for the treatment of industrial wastewater. In Proc. 39th Industrial Waste Conference. pp. 62-636. Indiana: Purdue University.

Madoni, P., Davoli, D., Gorbi, G., and Vescovi, L. 1996. Toxic effect of heavy metals on the activated sludge protozoan community. Water Research 30(1): 135-141.

Mahimairaja, S., and Bolan, N.S. 2004. Problems and prospects of agricultural use of distillery spentwash. In India, 3rd Australian New Zealand Soils Conference, 5-9 December 2004, University of Sydney, Australia [Online]. Available from: www.regional.org.au/au/asssi/

Mahler, H. R., and Cordes, E. H. 1966. Biological Chemistry. pp. 570. New York: Harper and Row.

Mane, J.D., Modi, S., Nagawade, S., Phadnis, S.P., and Bhandari, V.M. 2006. Treatment of spentwash using chemically modified bagasse and color removal studies. Bioresource Technolology 97: 1752-1755.

Manjinder, S.K., Harvinder, S.S., Deepak, K.S., Bhupinder, S.C., and Swapandeep, S.C. 2005. Comparative studies on potential of consortium and constituent pure bacterial isolates to decolorize azo dyes. Water Research 39: 5135-5141.

Martins, S., Jongen, W.M.F., and van Boekel, M. 2000. A review of maillard reaction in food and implications to kinetic modeling. Trends in Food Science and Technology 11: 364–373.

Matkar, L.S., and Gangotri, M.S. 2003. Acute toxicity tests of sugar industrial effluents on the freshwater crab, *Barytelphusa guerini* (H. Milne Edwards) (Decapoda, Potamidea). Pollution Research. 22: 269-276.

Meng, F., Chae, S.R., Drews, A., Kraume, M., Shin, H.S., and Yang, F. 2009. Recent advances in membrane bioreactors (MBRs): membrane fouling and membrane material. Water Research [Article In Press].

Metcalf & Eddy. 2004. Wastewater Engineering. Treatment and Reuse, pp. 545-1026. New York: Meg Grew-Hill Companies.

Miranda, P.M., Benito, G.G., Cristobal, N.S., and Nieto, C.H. 1996. Colour elimination from molasses wastewater by *Aspergillus niger*. Bioresource Technolology 57: 229-235.

Migo, V., Del Rosario, E.J., and Matsumuraz, M. 1997. Flocculation of Melanoidins Induced by Inorganic Ions. Journal of fermentation and bioengineering 83(3): 287-291.

Migo, V.P., Matsumura, M., Del Rosaria, E.J., and Kataoka, H. 1993. Decolorization of molasses wastewater using an inorganic flocculant. Journal of Fermentation and Bioengineering 75: 438–442.

Miyata, N., Iwahori, K., and Fujita, M. 1998. Manganese-independent and dependent decolourization of melanoidin by extracellular hydrogen peroxide and peroxidases from *Coriolus hirsutus* pellets. Journal of Fermentation and Bioengineering 85: 550-553.

Miyata, N., Mori, T., Iwahori, K., and Fujita, M. 2000. Microbial decolorization of melanoidin containing wastewaters: Combined use of activated sludge and fungus Coriolus hirutus. Journal of Fermentation and Bioengineering 89 (2): 145-150.

Mogens, H. 2002. Wastewater volumes and composition. (2nd ed.), Wastewater treatment: biological and chemical processes, pp. 11-42. New York: Springer.

Mohammad, P., Azarmidokht, H., Fatollah, M., and Mahboubeh, B. 2006. Application of response surface methodology for optimization of important parameters in decolorizing treated distillery wastewater using *Aspergillus fumigatus* UB2.60. International Biodeterioration and Biodegradation 57: 195-199.

Mohana, S. C., and Desai, D. 2007. Madamwar, Biodegradation and decolorization of anaerobically treated distillery spent wash by a novel bacterial consortium. Bioresource Technolology 98: 333-339.

Murata, M., Terasawa, N., and Homma, S. 1992. Screening of microorganisms to decolorize a model melanoidin and the chemical properties of a microbially treated melanoidin. Bioscience, Biotechnology, and Biochemistry 56: 1107-1182.

Nagano, A., Arikawa, E., and Kobayashi, H. 1992. The treatment of liquor wastewater containing high-strength suspended solids by membrane bioreactor system. Water Science and Technology 26(3-4): 887-895.

Nakajima-Kame, T., Shimomura, M., Nomura, N., Chanpornpong, T., and Nakahava, T. 1999. Decolorization of molasses wastewater by *Bacillus* sp. under thermophilic and anaerobic conditions. Journal of Bioscience and Bioengineering 87: 119-121.

Nandy, T., Shastry, S., and Kaul, S.N. 2002. Wastewater management in a cane molasses distillery involving bioresource recovery. Journal of Environmental Management 65: 25-38.

Nataraj, S.K., Hosamani, K.M., and Aminabhavi, T.M. 2006. Distillery wastewater treatment by the membrane-based nano-filtration and reverse osmosis processes. Water Research 40 (12): 2349-2356.

Ng, H.Y., Tan, T.W., Ong, S.L., Toh, C.A., and Loo, Z.P. 2006. Effects of solid retention time on the performance of submerged anoxic/oxic membrane bioreactor. Water Science and Technology 53(6): 7-13.

Nigam, P., Banat, I.M., and Marchant, R. 1996. Decolorization of effluent from the textile industry by a microbial consortium. Biotechnology Letters 18:117-20.

Nilsson, W.B., Paranjype, R.N., DePaola, L.A., and Strom, M.S. 2003. Sequence polymorphism of the 16S rRNA gene of Vibrio vulnificus is a possible indicator of strain virulence. Journal of Clinical Microbiology 41(1): 442-446.

OAE (Office of Agriculture Economics). 2004. Agricultural statistics of Thailand. Center for agricultural information, Office of agricultural economics. Ministry of Agriculture and Cooperatives, Bangkok, Thailand. Available from: http://www.oae.go.th/English/ index.htm

OAE (Office of Agriculture Economics). 2006. Agricultural Statistics of Thailand. Center for agricultural information, Office of Agricultural Economics, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. Available from: http://www.oae.go.th/English/ index.htm

OCSB (Office of the Cane and Sugar board). 2007. Molasses Production in Thailand Crops Year 1988/89-2006/07. Available from: http://en.ocsb.go.th/images/1194580942/ MPTCY1988892006071.pdf [2008, December 15]

Ohmomo, S., and others. 1987. Decolourization of molasses wastewater by a thermophilic strain *Aspergillus fumigatus* G-2-6. Agricultural and biological chemistry 51: 3339-3346.

Ohmomo, S., and others. 1988. Screening of anaerobic bacteria with the ability to decolourize molasses melanoidin. Agricultural and biological chemistry 57: 2429-2435.

Painter, T.J. 1998. Carbohydrate polymers in food preservation: an integrated view of the Maillard reaction with special reference to discoveries of preserved foods in Sphagnum-dominated peat bogs. Carbohydrate Polymers 36: 335-347.

Pala, A., and Erden, G. 2005. Decolorization of a baker's yeast industry effluent by fenton's oxidation. Journal of Hazardous Materials 127: 141-148.

Pandey, R.A., Malhotra, A., Tankhiwale, S., Pande, S., Pathe, P.P., and Kaul, S.N. 2003. Treatment of biologically treated distillery effluent a case study. International Journal of Environmental Studies 60: 263-275.

Pant, D., and Adholeya, A. 2007. Biological approaches for treatment of distillerywastewater: a review. Bioresource Technology 98: 2321-2334.

Patel, A., Pawar, P., Mishra, S., and Tewari, A. 2001. Exploitation of marine cyanobacteria for removal of colour from distillery effluent. Indian Journal of Environmental Protection 21: 1118-1121.

Patel, H. D. 2000. Madamwar, Biomethanation of low pH petrochemical wastewater using up-flow fixed film anaerobic bioreactors. World Journal of Microbiology and Biotechnology 16:69-75.

Patel, H., and Madamwar, D. 2002. Effects of temperatures and organic loading rates on biomethanation of acidic petrochemical wastewater using an anaerobic upflow fixed-film reactor. Bioresource Technology 82: 65-71.

Pathade, G.R. 2003. A review of current technologies for distillery wastewater treatment. In Goel, P.K. (ed.), Advances in Industrial Wastewater Treatment, pp. 180-239. Jaipur, India: ABD Publishers.

Pendyal, B., Johns, M.M., Marshall, W.E., Ahmedna, M., and Rao, R.M. 1999. Removal of sugar colorants by granular activated carbons made from binders and agricultural by-products. BioresourceTechnology 69: 45-51.

Pena, M., Coca, M., Gonzalez, R., Rioja, R., and Garcia, M.T. 2003. Chemical oxidation of wastewater from molasses fermentation with ozone. Chemosphere 51: 893-900.

Perez-Garcia, M., Romero-Garcia, L.I., Rodriguez-Cano, D., and Sales-Marquez. 2005. Effect of the pH influent conditions in fixed film reactors for anaerobic thermophillic treatment of wine-distillery wastewater. Water Science and Technology 51: 183-189.

Perez, M., Romero, L.I., Nebot, E., and Sales, D. 1997. Colonisation of a porous sintered glass support in anaerobic thermophilic bioreactors. Bioresource Technology 59: 177-183.

Petruccioli, M., Duarte, J.C., and Fedrerici, F. 2000. High rate aerobic treatment of winery wastewater using bioreactors with free and immobilized activated sludge. Journal of Bioscience and Bioengineering 90: 381-386.

Pikaev, A.K. 2001. New environmental applications of radiation technology. High Energy Chemistry 35: 148-160.

Plavsic, M., Cosovic, B., and Lee, C. 2006. Copper complexing properties of melanoidins and marine humic material. Science of the Total Environment 366: 310-319.

Raghukumar, C., Mohandass, C., Kamat, S., and Shailaja, M.S. 2004. Simultaneous detoxification and decolorization of molasses spentwash by the immobilized white-rot fungus *Flavadon flavus* isolated from the marine habitat. Enzyme and Microbial Technology 35: 197-202.

Raghukumar, C., and Rivonkar, G. 2001. Decolourization of molasses spent wash by the white-rot fungus *Flavodon flavus*, isolated from a marine habitat. Applied Microbiology and Biotechnology 55: 510-514.

Rajeshwari, K.V., Balakrishnan, M., Kansal, A., Lata, K., and Kishore, V.V.N. 2000. State-of art of anaerobic digestion technology for industrial wastewater treatment. Renewable and Sustainable Energy Reviews 4: 135-156.

Rao, S.B. 1972. A low cost waste treatment method for disposal of distillery waste (spent wash). Water Research 6: 1275-1282.

Rhoads, T.L., Mikell, A.T., and Eley, M.H. 1995. Investigation of the lignin-degrading activity of *Serratia marcescens*: biochemical screening and ultrastructural evidence. Canadian Journal of Microbiology 41:592-600.

Ross, B., and Strohwald, H. 1994. Membrane add edge to old technology. Water Quality International 4.

Rufián-Henares, J.A., and Morales, F.J. 2006. A new application of a commercial microtiter plate-based assay for assessing the antimicrobial activity of Maillard reaction products. Food Research International 39: 33-39.

Ruiz, M., and Rolz, C. 1971. Activated carbons from sugar cane bagasse. Industrial and Engineering Chemistry Product Research Development 10(4): 429-432.

Ruiz, C., Torrijos, M., Sousbie, P., Lebrato, J., Martinez, R., and Delgenes, J.P. 2002. Treatment of winery wastewater by an anaerobic sequencing batch reactor. Water Science and Technology 45: 219-224.

Sangave, P.C., and Pandit, A.B. 2004. Ultrasound pretreatment for enhanced biodegradability of the distillery wastewater. Ultrasonics Sonochemistry 11: 197-203.

Sarayu, M., Chiraya, D., and Datta, M. 2006. Biodegradation and decolourization of anaerobically treated distillery spent wash by novel bacterial consortium. Bioresource Technology 98: 333-339.

Sarayu, M., Shalini, S., Jyoti, D., and Datta, M. 2008. Response surface methodology for optimization of medium for decolorization of textile dye Direct Black 22 by a novel bacterial consortium. Bioresource Technology 99: 562-569.

Satyawali, Y., and Balakrishnan, M. 2007. Removal of color from biomethanated distillery spent wash by treatment with activated carbons. Bioresource Technology 98: 2629-2635.

Satyawali, Y., and Balakrishnan, M. 2008a. Wastewater treatment in molasses-based alcohol distilleries for COD and color removal: a review, Journal of Environmental Management. 86: 481-497.

Satyawali Y., and Balakrishnan, M. 2008b.Treatment of distillery effluent in a membrane bioreactor (MBR) equipped with mesh filter. Separation and Purification Technology 63 (2): 278-286.

Saxena, K.K., and Chauhan, R.R.S. 2003. Oxygen consumption in fish, *Labeo rohita* (HAM.) caused by distillery effluent. Ecology Environment and Conservation 357-360.

Senan, R.C., and Abraham, T.E. 2004. Bioremediation of textile azo dyes by aerobic bacterial consortium. Biodegradation 15(4):275-80.

Sharma, J., and Singh, R. 2000. Characterization of sludge from UASB reactors operating on molasses based distillery effluent. Indian Journal of Microbiology 40: 203-205.

Sharma, S., and others. 2007. Impact of distillery soil leachate on heamatology of swiss albino mice (*Mus musculus*). Bulletin of Environmental Contamination and Toxicology 79: 273-277.

Shayegan, J., Pazouki, M., and Afshari, A. 2004. Continuous decolorization of anaerobically digested distillery wastewater. Process Biochemistry 40: 1323-1329.

Singh, P.N., Robinson, T., and Singh, D. 2004. Treatment of industrial effluents distillery effluent. In Pandey, A. (ed.), Concise Encyclopedia of Bioresource Technology, pp. 135-141. New York: Food Products Press.

Singleton, I. 1994. Microbial metabolism of xenobiotics: fundamental and applied research. Journal of Chemical Technology and Biotechnology 59(1): 9-23.

Sirianuntapiboon, S., Phothilangka, P., and Ohmomo, S. 2004b. Decolourization of molasses wastewater by a strain no. BP103 of acetogenic bacteria. Bioresearch Technology 92: 31-39.

Sirianuntapiboon, S., and Prasertsong, K. 2008. Treatment of molasses wastewater by acetogenic bacteria BP103in sequencing batch reactor (SBR) system. Bioresource Technology 99: 1806-1815.

Sirianuntapiboon, S., Zohsalam, P., and Ohmomo, S. 2004a. Decolorization of molasses wastewater by *Citeromyces* sp. WR-43-6. Process Biochem 39: 917-924.

Skerratt, G. 2004. European distilleries: an overview. In Tewari, P.K. (ed.), Liquid Asset, Proceedings of the Indo-EU Workshop on Promoting Efficient Water Use in Agro-Based Industries, pp. 1-11. New Delhi, India: TERI Press.

Stephenson, T., Judd, S., Jefferson, B., and Brindle, K. 2000. Membrane Bioreactors for Wastewater Treatment. London: IWA Publishing.

Tay, J.H., Zeng, J.L., and Sun, D.D. 2003. Effects of hydraulic retention time on system performance of a submerged membrane bioreactor. Separation Science and Technology 38(4): 851-868.

Taylor, J.L.S., and others. 2004. Genotoxicity of melanoidin fractions derived from a standard glucose/glycine model. Journal of Agricultural and Food Chemistry 52: 318-323.

Thakkar, A.P., Dhamankar, V.S., and Kapadnis, B.P. 2006. Biocatalytic decolourisation of molasses by *Phanerochaete chrysosporium*. Bioresource Technology 97: 1377-1381.

The Excise Department. 1983. Sample quantities and characteristics of distillery slop generated in 32 Thai distilleries. Available from: http://www.exd.mof.go.th/ [2008, April 3].

Torrijos, M., and Moletta, R. 1997. Winery wastewater depollution by sequencing batch reactor. Water Science and Technology 35(1): 249-257.

Uzal, N., Gokcay, C.F., and Demirer, G.N. 2003. Sequential (anaerobic/aerobic) biological treatment of malt whisky wastewater. Process Biochemistry 39: 279-286.

Vahabzadeh, F., Mehranian, M., and Saatari, A.R. 2004. Colour removal ability of *Phanerochaete chrysosporium* in relation to lignin peroxidases and manganese peroxidases produced in molasses wastewaters. World Journal of Microbiology and Biotechnology 20: 859-864.

Valderrama, L.T., Del Campo, C.M., Rodriguez, C.M., Bashan, L.E., and Bashan, Y. 2002. Treatment of recalcitrant wastewater from ethanol and citric acid using the microalga *Chlorella vulgaris* and the macrophyte *Lemna minuscule*. Water Research 36: 4185-4192.

Valo, R., Apajahalati, J., and Salkinoja-Salonen, M. 1985. Studies on the physiology of microbial degradation of pentachlorophenol. Applied Microbiology and Biotechnology 21: 313-319.

Vlyssides, A.G., Israilides, C.J., Loizidou, M., Karvouni, G., and Mourafeti, V. 1997. Electrochemical treatment of vinasse from beet molasses. Water Science and Technology 36 (2-3): 271-278.

Vogelaar, J., Van Lier, J., Klapwijk, A., De Vries, M., and Lettinga, G. 2002. Assessment of effluent turbidity in mesophilic and thermophilic activated sludge reactors-origin of effluent colloidal material. Applied Microbiology and Biotechnology 59: 105–111.

Watanabe, Y., Sugi, R., Tanaka, Y., and Hayashida, S. 1982. Enzymatic decolourization of melanoidin by *Coriolus* sp. Agricultural and biological chemistry 46 (20): 1623-1630.

Wesenberg, D., Kyriakides, I., and Agathos, S.N. 2003. White-rot fungi and their enzymes for the treatment of industrial dye effluents. Biotechnology Advances 22: 161-187.

Wise, W. S. 1951. The measurement of the aeration of culture media. Journal of General Microbiology 5: 167-177.

Yang, W., Cicek, N., and Ilg, J. 2006. State-of-the-art of membrane bioreactors: worldwide research and commercial applications in North America. Journal of Membrane Science 207: 201-211.

Yaylayan, V.A., and Kaminsky, E. 1998. Isolation and structural analysis of Maillard polymers: Caramel and melanoidin formation in glycine/glucose model system. Food Chemistry 63: 25-31.

Yeoh, B.G. 1997. Two-phase anaerobic treatment of cane-molasses alcohol stillage. Water Science and Technology 36 (6-7): 441-448.

Yun, M.A., Yeon, K.M., Park, J.S., Lee, C.H., Chunb, J., and Lim, D.J. 2006. Characterization of biofilm structure and its effect on membrane permeability in MBR for dye wastewater treatment. Water research 40: 45-52.

Zhang, J. S., Chuan, C. H., Zhou, J. T. and Fane, A. G. 2006. Effect of sludge retention time on membrane bio-fouling intensity in a submerged membrane bioreactor. Separation Science and Technology 41(7): 1313-1329.

Zhang, S., Yang, F., Liu, Y., Zhang, X., Yamada, Y., and Furukawa, K. 2006. Performance of a metallic membrane bioreactor treating simulated distillery wastewater at temperatures of 30 to 45^{O}C. Desalination 194: 146-155.

APPENDICES

APPENDIX 1

Determination of chemical oxygen demand (COD)

COD was measured by Colorimetric determination using Reactor Digestion method (Hach COD reagent test kit, Hach Company, USA) as recommend by manufacturer.

Reagents

a. COD Digestion reagent vials (Hach COD reagent test kit; range 3 to 150 mg/L, 20 to 1500 mg/L) containing;

1. Potassium dichromate in sulfuric acid solution is a strong oxidizing agent.
2. Silver is a catalyst,
3. Mercury is used to complex Chloride interference

b. COD standard solution (Hach COD standard reagent)

Procedure

1. turn on the COD reactor and preheat the reactor to 150^{O}C.
2. add 2 ml of deionized water (blank) and samples to the COD digestion reagent vials.
3. tightly cap the vials and rinse them with deionized water. Then, wipe with a clean paper and invert gently each vial several times to mix completely. The sample vials will become very hot during mixing.
4. place the vial in the preheated COD reactor. Heat the sample at 150^{O}C for 2 hours.
5. turn off the COD reactor and wait about 20 minutes for the vials to cool to 120^{O}C or less. Invert gently each vial several times while still warm.
6. cool the vials to room temperature and place the vials in the test tube rack.
7. proceed to the colorimetric determination using Hach DR/2500 spectrophotometer (Hach Company, USA). Select the appropriate program for measuring the COD values. The programs are specified in Table11.1

Table A Method Performance

Program	Range in mg/L COD	COD Standard solution
430	3 to 150 mg/L	100 mg/L
435	20 to 1,500 mg/L	300 or 1,000 mg/L

Note: The colorimetric determination can measure COD value at the specific wavelengths which were specified in Table 11.2.

Table B The range-specific wavelengths

Range in mg/L COD	Wavelength
3 to 150 mg/L	420 nm
20 to 1,500 mg/L	620 nm

Determination of total nitrogen

Total Nitrogen was measured by Colorimetric determination using Persulfate digestion method (Test 'N Tube™ HR Total Nitrogen Reagent Set, Hach Company, USA) as recommend by manufacturer.

Reagents

a. Test 'N Tube™ HR Total Nitrogen Reagent Set containing: HR Total Nitrogen Hydroxide Digestion Reagent vials; range 10 to 150 mg/L, deionized water (free of all nitrogen-containing), Total Nitrogen Persulfate Reagent powder, Total Nitrogen (TN) Reagent A powder, TN Reagent B powder and TN Reagent C vials.

Procedure

1. turn on the COD reactor. Preheat to 103-106^{O}C.
2. add the contents of Total Nitrogen Persulfate Reagent powder to each of HR Total Nitrogen Hydroxide Digestion Reagent vials.
3. add 0.5 ml of deionized water included in the kit (blank) and sample to the vials. (Use only water that is free of all nitrogen-containing species as a substitute for the deionized water provided)
3. tightly cap the vials and shake vigorously for at least 30 seconds to mix.
4. place the vial in the preheated reactor and heat for 30 minutes.
5. remove the vials from the reactor and place in the test tube rack. Cool the vials to room temperature.
6. add the content of Total Nitrogen (TN) Reagent A powder to each vial.
7. cap the tubes and shake for 15 seconds. Then, stand the tubes for 3 minutes.
8. add TN Reagent B powder to each vial.
9. cap the tubes and shake for 15 seconds. Then, stand the tubes for 2 minutes.
10. add 2 ml of digested, treated samples (from item 9) to each TN Reagent C vial.
11. cap the vials and invert 10 times to mix. Then, stand the tubes for 5 minutes. The yellow color will intensify.
12. proceed to the colorimetric determination using Hach DR/2500 spectrophotometer (Hach Company, USA). Select the program 395N, Total HR TNT for measuring the total nitrogen values.

Note: The colorimetric determination can measure total nitrogen value at the specific wavelengths at 410 nm.

APPENDIX 2

The 16S rDNA sequences of bacteria

TGCTCTCGGG TGANGAGTGG CGGACGGGTG AGTAATGTCT GGGAAACTGC
CTGATGGAGG GGGATAACTA CTGGAAACGG TAGCTAATAC CGCATAACGT
CGCAAGACCA AAGAGGGGGA CCTTCGGGCC TCTTGCCATC AGATGTGCCC
AGATGGGATT AGCTAGTAGG TGGGGTAACG GCTCACCTAG GCGACGATCC
CTAGCTGGTC TGAGAGGATG ACCAGCCACA CTGGAACTGA GACACGGTCC
AGACTCCTAC GGGAGGCAGC AGTGGGGAAT ATTGCACAAT GGGCGCAAGC
CTGATGCAGC CATGCCGCGT GTATGAAGAA GGCCTTCGGG TTGTAAAGTA
CTTTCAGCGG GGAGGAAGGC GATNAGGTTA ATAACCTTGT CGATTGACGT
TACCCGCAGA AGAAGCACCG GCTAACTCCG TGCCAGCAGC CGCGGTAATA
CGGAGGGTGC AAGCGTTAAT CGGAATTACT GGGCGTAAGC GCACGCAGGC
GGTCTGTCAA GTCGGATGTG AAATCCCGGG CTCAACCTGG GAACTGCATN
CNAAACTGGC AGGCTGGANT CTTGTAAANG GGGNTAAANT CCNGGTGTAN
CGNTAAATGC NTAAAATCTG GAAGAATACC GGTGGCNAAG GGGCCCCCTG
GANAAAACTG ACCCTNAGTG CAAANCNTGG GGAACAANAG ATTAANANCC
CTGGNAAA

Figure A The partial 16S rDNA sequences of *Klebsiella oxytoca* (T1)

GGGACTTGCT CCCTGGGTGA NGAGCGGCGG ACGGGTGAGT AATGTCTGGG
AAACTGCCTG ATGGAGGGGG ATAACTACTG GAAACGGTAG CTAATACCGC
ATAACGTCGC AAGACCAAAG AGGGGGACCT TCGGGCCTCT TGCCATCAGA
TGTGCCCAGA TGGGATTAGC TAGTAGGTGG GGTAATGGCT CACCTAGGCG
ACGATCCCTA GCTGGTCTGA GAGGATGACC AGCCACACTG GAACTGAGAC
ACGGTCCAGA CTCCTACGGG AGGCAGCAGT GGGGAATATT GCACAATGGG
CGCAAGCCTG ATGCAGCCAT GCCGCGTGTG TGAAGAAGGC CTTCGGGTTG
TAAAGCACTT TCAGCGAGGA GGAAGGTGGT GAGCTTAATA CGNTCATCAA
TTGACGTTAC TCGCAGAAGA AGCACCGGCT AACTCCGTGC CAGCAGCCGC
GGTAATACGG AGGGTGCAAG CGTTAATCGG AATTACTGGG CGTAAAGCGC
ACGCCAGGCG GTTTGTTAAG TCANATGTGA AATCCCCGGN CTCAACCTGG
GAACTGCATT TTGAAACTGG CAAGCTANAG TCTCGTAAAG GGGGGTANAA
TTCCNGGTGT AACCGTGAAA TNCGTAAANA TCTGGANGAA TACCNGNTGG
NCAAAGGCGG CCCCCNTGGA CNAAAANNTN NCCTCAGGTN NNAAACCNNG
GGACCNNACN GNATTNATTC CCCTGGNNTN CCCNCCTTNA ANNNATGNCN
ANTTNNANGN TTGCCCCNTT NAGNCTNNT

Figure B The partial 16S rDNA sequences of *Serratia mercescens* (T2)

AGCAGCTTGC TGCTTCGCTG AACGAGTGGC GGACGGGTGA GTAATGTCTG
GGAAACTGCC CGATGGAGGG GGATAACTAC TGGAAACGGT AGCTAATACC
GCATAATGTC GCAAGACCAA AGAGGGGGAC CTTCGGGCCT CTTGCCATCG
GATGTGCCCA GATGGGATTA GCTTGTTGGT GAGGTAACGG CTCACCAAGG
CGACGATCCC TAGCTGGTCT GAGAGGATGA CCAGCCACAC TGGAACTGAG
ACACGGTCCA GACTCCTACG GGAGGCAGCA GTGGGGAATA TTGCACAATG
GGCGCAAGCC TGATGCAGCC ATGCCGCGTG TATGAAGAAG GCCTTCGGGT
TGTAAAGTAC TTTCAGCGGG GAGGAAGGTG TTGTGGTTAA TAACCGCAAC
AATTGACGTT ACCCGCANAA NAAGCACCGG CTAACTCCGT GCCAGCAGCC
GCGGTAATAC GGAGGGTGCA AGCGTTAATC GGAATTACTG GGCGTAAAGC
GCACGCAGNC GGTCTGTCAA GTCGNATGTG AAATCCCCGG GCTCAACCTG
GGAACTGCAT NCAAACTNGG CANGCTTGAN TCTCNTAAAA GGGGGNTAAA
ATTCCNGNTT TACNGNTNAA TTGCCTAAAA ATNTTGGAGN AAAACCNGTG
GNAAAGGCGC CCCCCTTGAA AAAAANTGAC CCTCANGTGC NAAACNTGGG
GAANAAAAAG ATNNANAANC CCCGGNTAAA A

Figure C The partial 16S rDNA sequences of *Citrobacter farmeri* (T3)

TTGCTCCTTG GGTGANGAGT GGCGGACGGG TGAGTAATGT CTGGGAAACT
GCCCGATGGA GGGGGATAAC TACTGGAAAC GGTAGCTAAT ACCGCATAAC
GTCGCAAGAC CAAAGAGGGG GACCTTCGGG CCTCTTGCCA TCGGATGTGC
CCAGATGGGA TTAGCTAGTA GGTGGGGTAA CGGCTCACCT AGGCGACGAT
CCCTAGCTGG TCTGAGAGGA TGACCAGCCA CACTGGAACT GAGACACGGT
CCAGACTCCT ACGGGAGGCA GCAGTGGGGA ATATTGCACA ATGGGCGCAA
GCCTGATGCA GCCATGCCGC GTGTATGAAG AAGGCCTTCG GGTTGTAAAG
TACTTTCAGC GAGGAGGAAG GNGTTGTGGT TAATAACCGC ANCGATTGAC
GTTACTCGCA GAAGAAGCAC CGGCTAACTC CGTGCCAGCA GCCGCGGTAA
TACGGAGGGT GCAAGCGTTA ATCGGAATTA CTGGGCGTAA AGCGCACGCA
GGCGGTCTGT CAAGTCNGAT GTGAAATCCC CGGGCTCAAC CTGGGAACTG
CATCCGAAAC GGCAGGCTAG AGTCTTGTAN AGGGGGGTAA ANTCCAGGTG
TAGCGGTGAA ATGCGTAAAN ATCTGGAAGA ATACCGGTGG CNAANGCGNC
CCCCTGGANA AAACTGACNC TCANGTGCNA AAGCGTGGGG AACAACAGNN
TNAAANNNCC CCNGGNAAAA

Figure D The partial 16S rDNA sequences of Unknown bacterium (T4)

APPENDIX 3

Experimental Techniques for k_La determination

The K_La is measure of quantity of oxygen that can actually be transferred into a volume element, and it characterizes the efficiency of oxygen transfer.

Theory:

$$\frac{dC_{AL}}{dt} = k_L a\left(C^*_{AL} - C_{AL}\right)$$

Integrating….

$$\int_{C_{AL1}}^{C_{AL2}} \frac{1}{\left(C^*_{AL} - C_{AL}\right)} dC_{AL} = k_L a \int_0^t dt$$

$$\ln\left(\frac{C^*_{AL} - C_{AL1}}{C^*_{AL} - C_{AL2}}\right) = k_L a t$$

Therefore a plot of $\ln\left(\frac{C^*_{AL} - C_{AL1}}{C^*_{AL} - C_{AL2}}\right)$ versus **t** should result in a straight line of slope K_La.

C_{AL} = Dissolved oxygen concentration in the broth (mMol O_2 L^{-1})
C^* = Saturated oxygen concentration in the broth (mMol O_2 L^{-1})
K_La = Oxygen transfer coefficient (h^{-1})
t = time (h)

KLa is measured by Static Gassing out method (Wise, 1951)

Procedure

1. In the absence of respiring organism (no O_2 consumption). Sparge vessel contents with N_2 to displace O_2. Monitor variation in dissolved oxygen concentration (DO) using a DO probe. Allow DO to fall to 0% saturation, then turn off N_2 flow

2. Sparge vessel contents with air at a known flow rate. Monitor and record variation of DO concentration with respect to time.

3. Plot of $\ln\left(\frac{C^*_{AL} - C_{AL1}}{C^*_{AL} - C_{AL2}}\right)$ versus **t** should result in a straight line of slope $k_L a$

Oxygen trasfer in melanoidins-containing wastewater medium

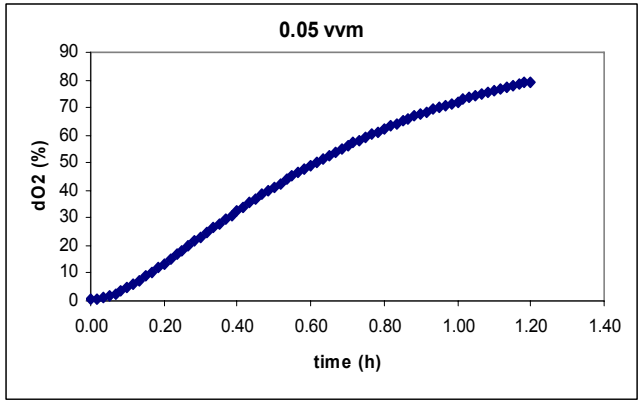


Figure E DO concentration with respect to time at 0.05 vvm

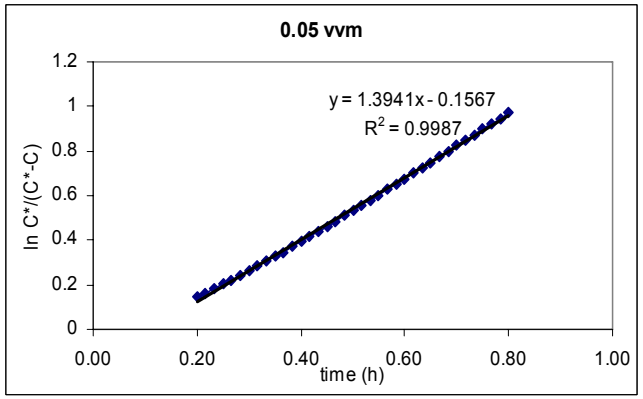
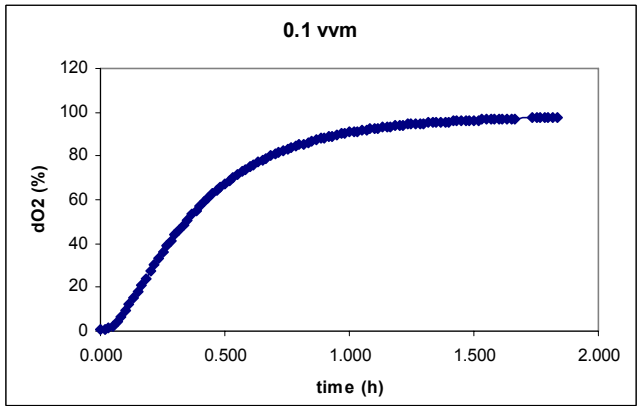


Figure F ln C*/(C*-C) value with respect to time at 0.05 vvm



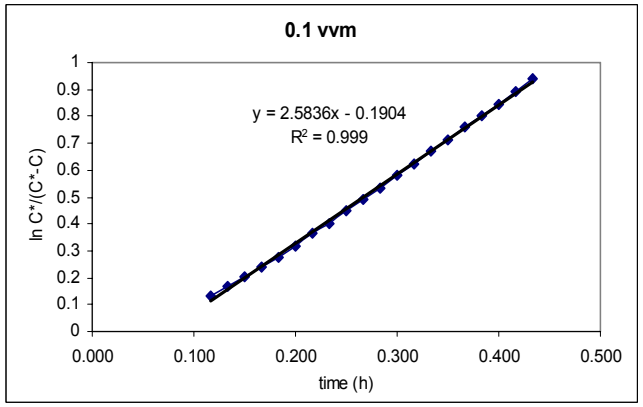


Figure G DO concentration with respect to time at 0.1 vvm

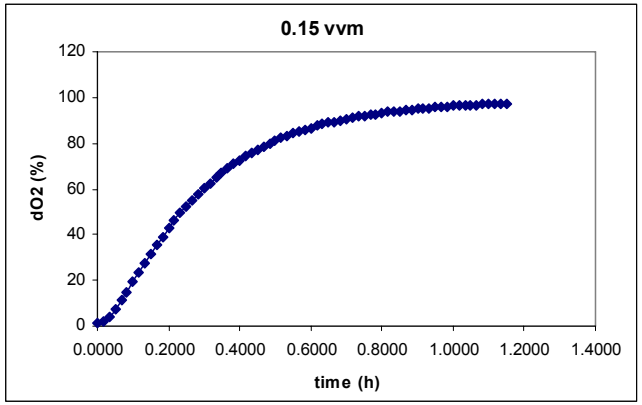


Figure H ln C*/(C*-C) value with respect to time at 0.1 vvm

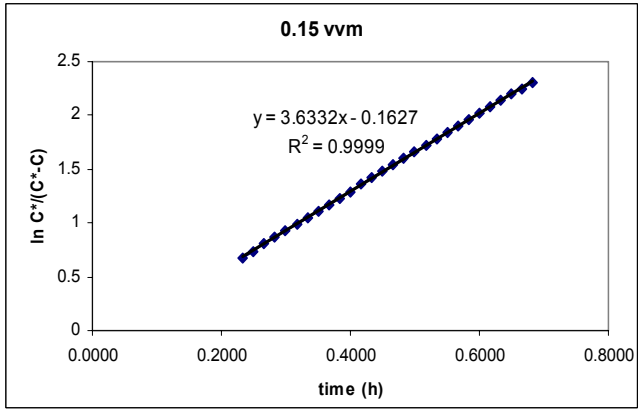
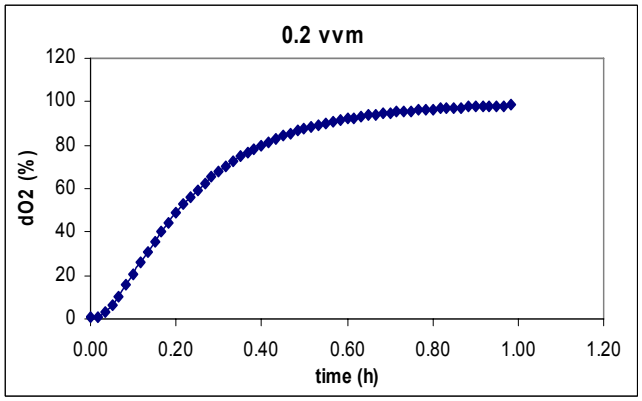


Figure I DO concentration with respect to time at 0.15 vvm

Figure J ln C*/(C*-C) value with respect to time at 0.15 vvm



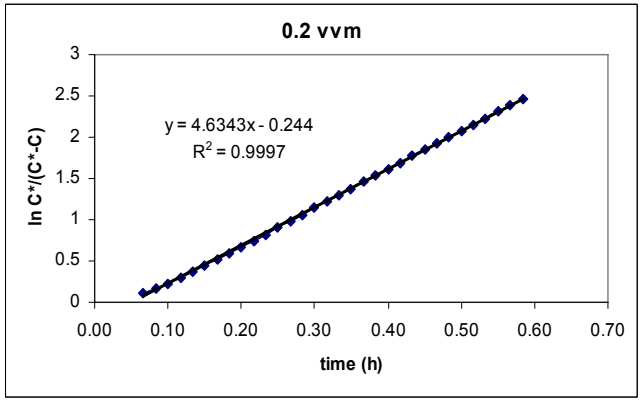


Figure K DO concentration with respect to time at 0.2 vvm

Figure L ln C*/(C*-C) value with respect to time at 0.2 vvm

Appendix 4

Method for testing membrane permeability

The test of membrane permeability was conducted under controlled laboratory conditions with the temperature of 25°C. The membrane module was a stainless-steel monochannel microfiltration module, a mineral M14 Carbosep® membrane. This membrane module was equipped separately with a bioreactor tank having a working volume of 1.6L. The reactor was filled with double distilled water (DDW). A peristaltic pump (Master Flex I/P) was used to supply the operating pressure and DDW circulation through the membrane module. A water level was used to control the influent pump and to keep the water level in the bioreactor constant.

The membrane permeability was determined by measuring the variation of permeate flux at different transmembrane pressure (TMP), which adjusted by stepwise increasing pump rotational speed from 50 to 750 rpm. At different pump speed, permeate flux through the membrane were recorded. During the experiment, the withdrawn water was returned to the tank to keep the water level in the tank constant. A plot of permeate flux (J) versus transmembrane pressure (represent by pump speed) should result in a straight line of slope permeability. Therefore, if the slope of the permeability curve is changed, then permeability of a given membrane is changed. Figure M shows the example of water permeability of the mineral membrane before and after operation.

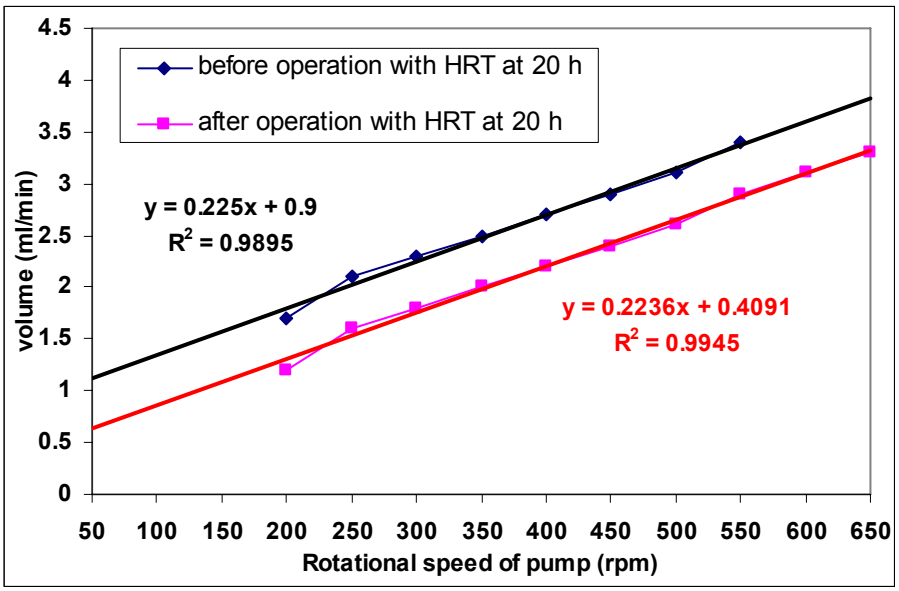


Figure M Membrane permeability of the mineral membrane before and after operation

Theory:

The relationship between the permeate flux and transmembrane pressure is given in the following equation:

$$J = \frac{\Delta P}{\mu R_t} \qquad \text{Eq. (1)}$$

where J = Equilibrium permeate flux (L/m^2.h)

ΔP = Transmembrane pressure, TMP (kPa)

μ = Viscosity of the permeate (kN.s /m^2)

Rt = Total membrane resistance

$$R_t = R_m + R_f = R_m + R_c + R_p \qquad \text{Eq. (2)}$$

R_m = Intrinsic membrane resistance,

R_c = Cake resistance,

R_f = Fouling resistance and

R_p = Resistance due to pore plugging and irreversible fouling.

Permeate flux and TMP data were used to calculate resistances by Eq. (1); filtration of pure water with a new membrane before operation gives the R_m, and R_t was calculated from the final flux and TMP values at the end of the operation.

$R_m + R_p$ is measured after removing the cake layer by washing the membrane with water after the operation followed by filtration with pure water. From these values each of R_t, R_m, R_c, R_p and R_f can be obtained using Eq. (2).

BIOGRAPHY

Miss Suhuttaya Jiranuntipon was born on July 5, 1976 at Bangkok, Thailand. She has graduated and holds a B.Sc. in Microbiology from Department of Microbiology, Faculty of Science, Chulalongkorn University in 1996. She has graduated and holds a M.Sc. in Industrial Microbiology from Department of Microbiology, Faculty of Science, Chulalongkorn University in 2001. She has applied to study Ph.D. in Biotechnology at the Program in Biotechnology, Faculty of Science, Chulalongkorn University in 2004. The Royal Golden Jubilee Program of the Thailand Research Fund and Embassy of France in Thailand financially supported her Ph.D. program.